University of Alberta

The Characterization of TSH-mediated Phospholipase D Activity in Thyroid Cells

by

Marni Allison Devlin ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Medicine

Department of Medicine

Edmonton, Alberta
Fall 1999

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Marni A. Devlin

June 25/99

Bsmt. 10686-63 Ave.
Edmonton, AB
T6H 1P6

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Characterization of TSH-mediated Phospholipase D Activity in Thyroid Cells submitted by Marni Allison Devlin in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

Dr. Jody Ginsberg

Dr. David N. Brindley

Dr. Luc Berthiaume

Dr. Alan B.R. Thomson

Dr. Charles Holmes

22/6/99

To the two people who made my life possible,

Mom and Dad

ABSTRACT

Phospholipase D (PLD) has emerged as a possible regulator of thyroid function. Thyrotropin (TSH) action occurs through the activation of two distinct pathways, one involving protein kinase A (PKA) and the other protein kinase C (PKC), which appear to converge on PLD activation. Two mammalian phosphatidylcholine-specific PLDs exist, PLD1 and PLD2. PLD1 requires small G-proteins and guanosine 5'-O-(3-thiotriphosphate) (GTPγS) for activity while PLD2 appears largely independent of these stimuli. In FRTL-5 thyroid cells, GTPγS stimulated the membrane-bound PLD maximally in the presence of cytosolic factors, suggesting the involvement of small G-proteins as well as the predominance of the PLD1 isoform. In addition to the characterization of PLD isoform composition, the ability of TSH to stimulate each of these isoforms was investigated. TSH activates PLD1 through translocation of the G-proteins ARF and RhoA to the membrane fraction. Our results suggest that PLD1 plays an important role in TSH-mediated events in FRTL-5 thyroid cells and that it could be an important regulator of thyroid hormone production.

ACKNOWLEDGMENT

I would like to express my sincerest gratitude to the following people:

Dr. Jody Ginsberg my supervisor.
Thank you for giving me the opportunity to experience science with all its rewards and difficulties. Your guidance and support have given me the confidence to explore new areas of interest and your encouragement has helped me to realize my potential. I also greatly appreciate your critical review of my thesis.

Dr. David N. Brindley my co-supervisor.
Thank you for keeping your door open and allowing me the chance to exercise my scientific mind. Your positive reinforcement has given me the drive to complete my studies and your scientific knowledge has given me the tools to expand my mind. I am also thankful for your thorough review and suggestions to my thesis.

From the Brindley lab.
Indrapal Singh - Thank you for the countless hours you spent teaching and helping me to understand not only how to do experiments, but also how to think scientifically.
Atef Hanna - Thank you for introducing me to the Western blot and ensuring that my results were as good as they could be.
Jay Dewald - Thank you for showing me how to do just about everything right from the first moment I arrived.

Thank you to Audric Moses, Ryan Perry, Vic Drover and Sheldon Radford who have assisted me with computer-related inquiries and have helped make my days more interesting.

A special thanks to several people from down the hall who have helped me understand what it is to be a graduate student and for making my days a little brighter, Russ Watts, Scot Stone, and Agnes Kulinski.

Thank you to many people from the lipid and lipoprotein research group who have all been more than willing to help whenever I needed it. Thanks also to Saswati Das for help in the lab.

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LIST OF ABBREVIATIONS

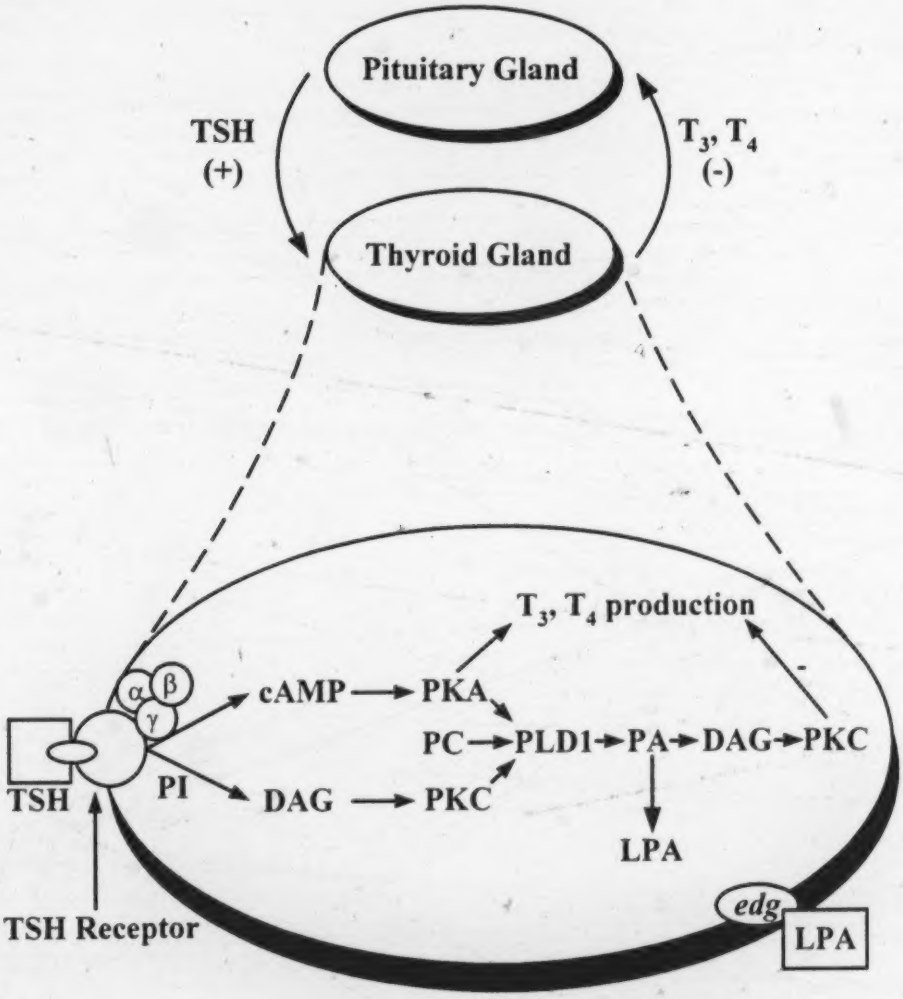
ARF	ADP-ribosylation factor
ARP	ADP-ribosylation factor-related protein
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cdc	cell division cycle
Chel	chelerythrine
Ci	Curie
CTA	cholera toxin A
DAG	diacylglycerol
dbcAMP	dibutyryl cyclic adenosine monophosphate
DDA	dideoxyadenosine
DMSO	dimethylsulfoxide
dpm	disintegrations per minute
DTT	dithiothreitol
ECL	enhanced chemiluminescence
edg	*endothelial differentiation gene*
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
EGTA	ethyleneglycol bis-(β-aminoethyl ether) -N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
ERM	erzin/radixin/moesin
FIG.	figure
fMLP	f-Met-Leu-Phe
FRTL-5	Fischer rat thyroid line - 5
g	gram
G-proteins	guanine nucleotide dependent proteins
GAP	GTPase activating protein
GDPβS	guanosine 5'-O-(2-thiodiphosphate)
GDI	GDP dissociation inhibitors
GEF	guanine nucleotide exchange factors
GTPγS	guanosine 5'-O-(3-thiotriphosphate)

HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'2-ethanesulfonate
hnRNPA1	heterogeneous ribonucleoprotein A1
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
kDa	kilodaltons
KH_2PO_4	potassium dihydrogen phosphate
l	litre
LPA	lysophosphatidic acid
LPP	lipid phosphate phosphohydrolase
M	molar
MAPK	mitogen activated protein kinase
MARCKS	myristolated alanine-rich C-kinase substrate
$MgCl_2$	magnesium chloride
min	minute
MW	molecular weight
NaCl	sodium chloride
NADPH	nicotinamide
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidate phosphohydrolase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PEt	phosphatidylethanol
PH	pleckstrin homology
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3' kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-1,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C

PLD	phospholipase D
PMA	phorbol myristate acetate
PS	phosphatidylserine
PX	phox
rpm	revolutions per minute
SAPK	stress-activated protein kinase
SD	standard deviation
SDS	sodium dodecyl sulfate
SRF	serum response factor
T_3	triiodothyronine
T_4	thyroxine
TLC	thin layer chromatography
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octyl phenoxypolyethoxyethanol
TSH	thyrotropin
vzg-1	*ventricular zone gene-1*

1. INTRODUCTION

PROPOSED MODEL OF TSH ACTION



1.1 THYROID

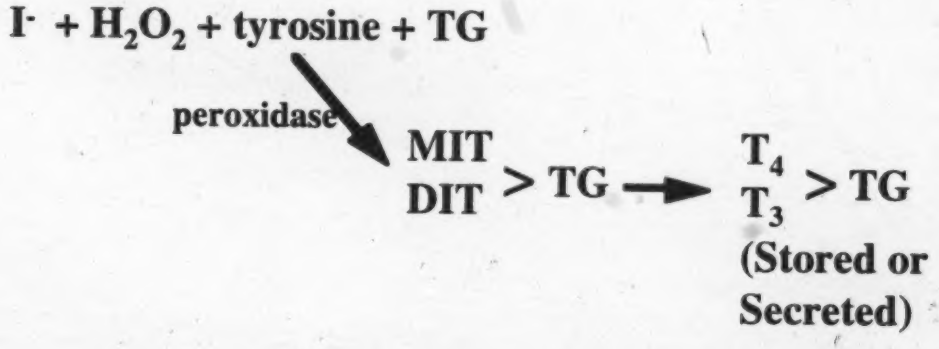
The thyroid gland produces hormones which are involved in the growth and metabolism of almost every tissue in the body (1). Thus, thyroid disease can affect almost every organ system. The synthesis and release of thyroid hormones is controlled by pituitary thyrotropin (TSH) via its membrane receptor (1). Thyroid hormones themselves have a negative feedback signal on the secretion of TSH by the pituitary (2). Follicular cells of the thyroid gland produce two major hormones, triiodothyronine (T_3) and thyroxine (T_4). These are the hormones involved in growth and metabolism throughout the body.

1.2 THYROID DISEASE

Thyroid disease affects about 2-5% of the North American population. TSH production and release is a target of subsequent negative feedback from thyroid hormones and, thus, is a critical aspect of thyroid regulation. Autoimmune diseases of the thyroid involve the inappropriate production of thyroid hormone via circulating autoantibodies (3) directed at the TSH receptor. The treatment of some thyroid diseases, like goiter and thyroid cancer, involves the suppression of TSH using exogenous L-thyroxine. Thus, TSH is the primary mediator of thyroid hormone production, release and growth. Graves' disease is an autoimmune disorder where there is a circulating antibody to the TSH receptor which increases thyroid hormone production independent of TSH (4,5). This TSH receptor antibody is not subject to negative feedback regulation. This is one example of thyroid disease which can be manifested in a number of ways resulting in mild to severe symptoms. The need to clearly establish the pathways by which thyroid hormones are produced is critical to our understanding and treatment of thyroid disease.

1.3 THYROID HORMONE SYNTHESIS

Thyroid hormone production by TSH involves a complex series of steps following receptor activation. The thyroid gland concentrates iodide from blood into thyroid follicular cells. Iodide taken up by the thyroid is oxidized by H_2O_2 in the presence of peroxidase forming a high energy molecule capable of binding enzymatically to tyrosyl residues of the thyroglobulin (TG) molecule, a process known as organification (6). The iodinated amino acids can have either one or two sites of iodide incorporation giving rise to monoiodotyrosine (MIT) or diiodotyrosine (DIT). The coupling of these molecules with either one of each type or two DITs gives rise to the thyroid hormones T_3 and T_4 which are stored in colloid surrounded by the follicular cells of the thyroid gland. When thyroid hormone is required, pseudopods extend from the follicular cell into the colloid, fuse with a phagosome and release thyroid hormone into circulation.



The production and release of thyroid hormones is the result of stimulation of thyroid cells by TSH. However, the intracellular pathway leading to thyroid hormone synthesis is complex and involves several intermediates which will be discussed in the following sections.

1.4 FRTL-5 THYROID CELLS

In order to provide a better understanding of the pathways involved in thyroid hormone synthesis, a rat thyroid cell model responsive to TSH was used. The cells used in our lab are called Fischer Rat Thyroid Line-5 (FRTL-5) cells. These are epithelial cells derived from normal rat thyroid grown in six hormone media containing TSH until confluence is reached, and then switched to five hormone media lacking TSH (7). These cells can then be used to study TSH action in thyroid cells. There are both advantages and disadvantages to the use of this model system. Some limitations to the use of these cells include the fact that they are grown long term in the presence of chronic TSH stimulation which could affect their subsequent response. Another drawback to the use of these cells is that they do not undergo iodide organification (7), a necessary step in thyroid hormone production, which makes it difficult to conclude that changes in early events (ie. cAMP generation or iodide uptake) will eventually result in increased thyroid hormone synthesis. However, there are several advantages to the use of FRTL-5 cells including the presence of a TSH receptor whose binding characteristics are similar to the human TSH receptor which makes it a good model for study in relation to human disease. In addition, these cells grow well in a hormonally and chemically defined media and have genetic homogeneity which is useful in defining specific actions in thyroid cells. Overall, FRTL-5 thyroid cells provide a practical working model for the study of TSH action which can be easily assessed and modified.

1.5 TSH RECEPTOR

The action of TSH on the thyroid gland is mediated by its interaction with the TSH receptor. The TSH receptor is a glycosylated hormone receptor (8) that is G-protein coupled (9). Initial studies of the TSH receptor revealed the presence of two subunits that were labeled A and B. It was proposed that the A subunit interacted with TSH and the B subunit anchored the receptor in the basolateral membrane of thyroid

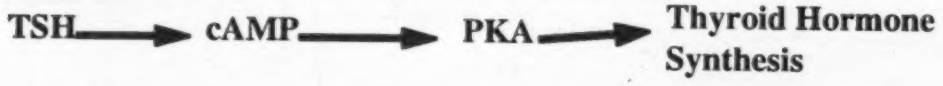
cells (8). Although cloning of the TSH receptor proved difficult, it was eventually accomplished using homologous hormone receptors, FSH and LH/CG, as probes (10-14). The TSH receptor is encoded by a single mRNA (15). Thus, the subunits identified by previous studies were the result of an intramolecular cleavage event of the polypeptide chain (16). However, both the cleaved and uncleaved TSH receptor exist in thyroid cells, although the reason for this is unknown (17). In addition, there is not one single cleavage site giving rise to the A and B subunits. There are two cleavage sites which result in the formation and release of a small polypeptide chain referred to as the C subunit (18). The function of the C subunit is unknown. The structure of the TSH receptor resembles that of other 7-transmembrane peptide hormone receptors with a considerable amount of homology with other glycoprotein hormone receptors (14,19). Thus, the structure-function relationship of those receptors was applied to formulate models of TSH receptor function.

Several hormone receptors, including the TSH receptor, were found to be single polypeptide chains with their amino terminus and carboxy terminus in the extracellular and intracellular space, respectively, with the middle portion of the protein traversing the membrane seven times (20,21). The TSH receptor shares some common characteristics with gonadotropin receptors in that the precursors of the receptors contain a signal peptide directing them to their location in the cell membrane and have long extracellular amino terminal domains (14,15,19). This amino terminal domain has a conserved leucine-rich sequence which has been implicated in protein-protein or protein-membrane interactions (22). Since there is significant homology between the carboxy termini of these different receptors, it was proposed that the amino terminus would confer hormone specificity to each receptor (15,16). In addition, since the TSH receptor is G-protein coupled, the activation of adenylyl cyclase by the $G\alpha$ subunit was expected. This was confirmed in thyroid cells where TSH was able to activate adenylyl

cyclase (15). The TSH receptor structure/function is important to our understanding of how TSH is able to elicit its effects within the thyroid cell.

1.6 TSH ACTION

It has been well established that TSH binding and stimulation of the TSH receptor leads to the activation of adenylyl cyclase and the accumulation of cyclic adenosine monophosphate (cAMP). Cyclic AMP then activates its respective protein kinase A (PKA), which ultimately leads to increases in thyroid hormone synthesis (15) via steps outlined earlier.



Although this pathway has been well characterized, without further intracellular regulation, unchecked thyroid hormone production would occur. This prompted the search for other intracellular signals that could inhibit this pathway, and one which was also activated by TSH.

The first report that TSH stimulated a cAMP-independent phosphoinositide (PI) turnover (23) occurred in 1953 by Morton and Schwartz. This was the first demonstration of a pathway other than cAMP that was activated by TSH in thyroid cells. In other model systems, a cAMP-independent pathway involving PI turnover, diacylglycerol (DAG) accumulation, intracellular Ca^{2+} increase and protein kinase C (PKC) activation has been subsequently observed (24,25). Thus, the role of PKC in TSH-mediated events was considered and studied in relation to thyroid function. Phorbol esters can activate PKC *in vitro* and *in vivo* as they contain a side chain similar to the established activator of PKC, DAG (26). Although the effects of phorbol esters on differentiated thyroid function *in vitro* vary according to concentration, incubation time, species and tissue expression, it was initially shown that they cause an inhibition of iodide uptake and organification in TSH-stimulated ovine thyroid cells (27). This

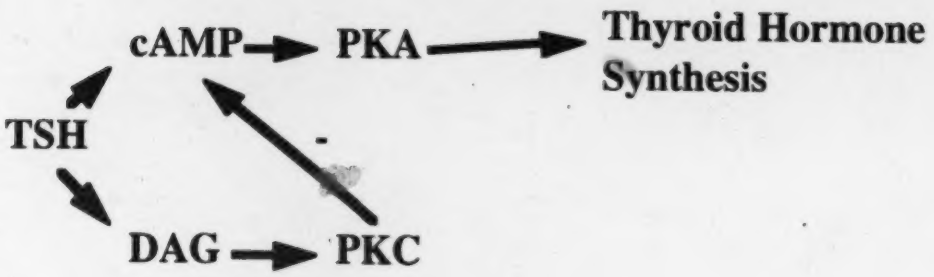
was an interesting observation linking the regulation of thyroid function to a pathway independent of cAMP. The addition of a cAMP analogue to this system did not recover iodide metabolism which implies that the site of phorbol ester action involves a post-receptor locus (27). Similar results were obtained using porcine (28), canine (29), and FRTL-5 thyroid cells (30). Some studies with mouse thyroid lobes demonstrated an increase in T_3 production with low phorbol ester concentrations (31) and others showed increases in basal iodide organification after phorbol ester stimulation (32-35). However, these observations may be species-specific and are inconsistent with other findings. In addition, phorbol esters inhibited TSH and forskolin-mediated cAMP generation in ovine (27) and FRTL-5 (30) thyroid cells, although the inhibition of iodide organification is not fully explained by this effect. In other cells, phorbol esters had no effect on cAMP levels (32,33,36) and, in some, a significant inhibition of TSH-stimulated iodide organification was observed (36). Despite these contradictory findings, using dog (35) and porcine (34) thyroid cells, short term phorbol ester exposure produced no effect on cAMP, but prolonged exposure produced an inhibition. This may indicate a temporal effect of phorbol esters on cAMP in some cells and some studies may not have included these longer time periods to observe this effect. Thus, the majority of studies indicated an inhibitory effect of prolonged phorbol ester treatment on thyroid hormone synthesis.

The mechanism of phorbol ester action and inhibition of thyroid hormone synthesis was investigated. Although phorbol esters have been shown to affect epidermal growth factor (EGF) binding to its receptor (37), this action did not explain their effects on TSH-mediated events. In addition, they do not appear to interact with TSH binding to its receptor in thyroid cells (27,36). Since the action of phorbol esters did not appear to involve the inhibition of TSH binding to its receptor, their ability to modulate post-receptor events was studied. Since phorbol esters resemble DAG, it was suggested that they act by activating PKC in the cell (26). However, some observed

effects of phorbol ester were not mediated by PKC (38). In Friend erythroleukemia cells, the suppression of the globin gene by phorbol ester occurs in spite of the down regulation of PKC (39). In addition, in ovine and porcine thyroid cells, a DAG analogue cannot fully mimic the effects of phorbol ester on iodide organification *in vitro* (27,28). However, the action of phorbol esters on the thyroid cell appears to be via PKC activation (25). Phorbol esters can activate PKC in porcine thyroid cells simultaneous with iodide organification inhibition (34). Non-phorbol PKC activators, like mezerein (40), have been shown to parallel the inhibitory effects of phorbol esters on differentiated thyroid function (30,36). TSH was also able to translocate PKC to the membrane in porcine thyroid cells (28). The use of PKC inhibitors reverses the inhibitory effects of phorbol esters on differentiated thyroid function *in vitro* (41,42). PKC in the thyroid has been shown to play a role in increasing H_2O_2 generation (43), glucose oxidation (44), Na^+/H^+ exchange (45), oncogene formation (29,46) and cell growth (27,29,30,45). The role that these processes play in regulating thyroid hormone synthesis is under investigation. However, H_2O_2 generation is involved in iodide organification in thyroid hormone synthesis (47). Thus, PKC could be an important mediator of differentiated thyroid function.

1.7 PROTEIN KINASE C IN THE THYROID

The search for the cAMP-independent pathway regulating thyroid hormone production led to the discovery of PKC involvement. TSH has been shown to cause the accumulation of diacylglycerol (DAG) as a consequence of activation of the phosphatidylinositol (PI) cascade (48). Since DAG is an activator of some PKC isoforms in many cell types, this action was identified in FRTL-5 thyroid cells (49,50). This new pathway involving PKC appeared to have an inhibitory effect on thyroid hormone synthesis, possibly through an inhibition of cAMP production (48).



However, in this model, the inhibition occurs before the increase in thyroid hormone synthesis which is a bit of a paradox. Therefore, there must be additional components in this pathway mediating the effects of both the cAMP/PKA and PKC pathways. In other cell types, the activation of PKC leads to PLD stimulation (51). This possibility was tested in FRTL-5 thyroid cells and studies showed that PLD was activated by activators of PKC (52,53). However, the role that PLD plays in terms of interacting with PKA and PKC remained unknown.

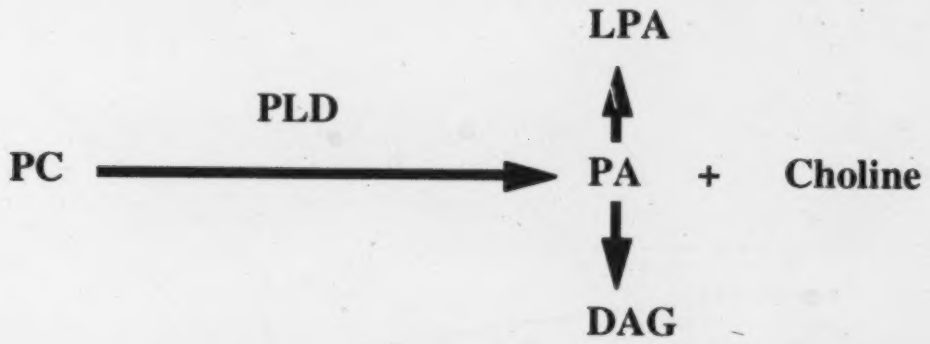
1.8 PROTEIN KINASE A IN THE THYROID

The role of PKA in mediating the effects of PLD has not been studied in much detail because, in most cells, PKA does not appear to play a role in PLD activation. In rat hepatocytes, a PKA-mediated pathway was able to increase PLD activity after vasopressin stimulation, although the mechanism for this activation appeared to involve an increase in intracellular Ca^{2+} levels (54). Thus, our demonstration of cAMP/PKA activation of PLD in FRTL-5 thyroid cells is unique (55). In FRTL-5 thyroid cells, TSH leads to PKA activation which is involved in the production of thyroid hormones (15). PKA-mediated PLD activation occurs independently of PKC (55). Although these results differ from those observed in other cell systems, they are consistent with similar findings in hepatocytes (54). As described in section 1.5, stimulation of the TSH receptor with agonist leads to the activation of adenylyl cyclase and the accumulation of cAMP, which activates PKA. This pathway leading to thyroid

hormone production has been well characterized in thyroid cells for many years. The chronology of PKA and PKC involvement in PLD activation will require additional study. Overall, PLD involvement in thyroid function was a new idea and one which required further understanding.

1.9 PHOSPHOLIPASE D

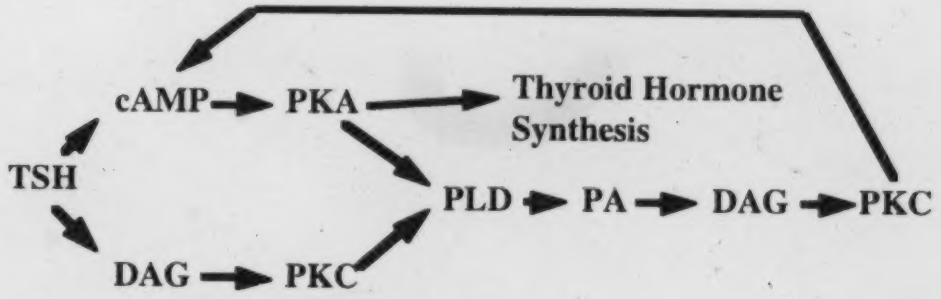
Phospholipase D (PLD) is widely distributed in mammalian cells (56) but its biological role has not been clearly defined. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) releasing a choline moiety by attacking the terminal phosphodiester bond of PC (51). The reaction is as follows:



The product of PLD hydrolysis of PC, PA, is a known intracellular signalling molecule in many cell systems and is involved in several signal transduction pathways. PA itself can be converted to lysophosphatidic acid (LPA) or DAG which also have signalling properties. PA has been demonstrated to be involved in the inhibition of adenylyl cyclase (57), increasing superoxide formation (58,59), and stimulation of mitogen-activated protein kinase (MAPK) (60); while LPA has roles in cell proliferation (61-63), platelet aggregation (61), and stress fiber formation (61). DAG acts via its activation of PKC isoforms (64). Thus, PLD may be an important mediator of these functions and PA could be mediating PLD's effects in thyroid cells. The specific roles of DAG and LPA will be discussed in sections 1.13 and 1.15 respectively. Other roles

for PLD may involve the initiation of transcription of certain genes in the endoplasmic reticulum (ER) and Golgi involved in the folding of proteins and secretion (65-67). Further evidence for this comes from studies showing that PA is necessary for the transport of proteins from the ER to the Golgi in mammalian cells (68). In some cells, PLD can catalyze the hydrolysis of phosphatidylethanolamine (PE) or phosphatidylinositol (PI), liberating the ethanolamine or inositol respectively and, again, PA (69). Although this thesis will focus on PLD hydrolysis of choline, it is important to note that different forms of PLD have the ability to carry out this differential hydrolysis. The role of PLD in cells appears to be the modification of the lipid composition of the plasma membrane to cause the activation of signal transduction pathways.

PLD is an enzyme found mainly in the membrane fraction of cells. In thyroid cells, a specific role for PLD has yet to be determined. In order to establish PLD as a mediator of both the PKA and PKC pathways, we formulated a model by which TSH could act in the thyroid cell.



In this model, both the PKA and PKC pathways converge on the activation of PLD. As stated above, PLD action leads to the production of PA. PA is rapidly converted to DAG by phosphatidate phosphohydrolase or lipid phosphate phosphohydrolase (LPP) (70), which also has a number of effects in the cell including the activation of conventional and novel PKC isoforms (71,72). Studies have shown that PKC is an

inhibitor of thyroid hormone synthesis (48), possibly through the inhibition of cAMP production, as discussed in section 1.7. However, as noted before, an unregulated PKC signal prior to PLD activation would be a poor candidate for the "inhibitory pathway" as it occurs before the stimulation of thyroid hormone synthesis. Thus, our model proposes that different PKC isoforms are involved at different times in this signal transduction pathway to elicit appropriate chronological responses in the cell. The role of specific PKC isoforms has not yet been studied in thyroid cells in relation to their respective functions.

Activators of PLD are still being defined, however, some of these have been characterized. Previous studies have shown that guanosine 5'-O-(3-thiotriphosphate) (GTPγS) can activate PLD in a Ca^{2+} -dependent manner in human neutrophils or Chinese hamster lung fibroblasts (73,74). This PLD activation, which has been observed in an *in vitro* system, requires the presence of both membranes and cytosol for maximum activity which indicates that components of both fractions are necessary for PLD activation (73-75). Small molecular weight G-proteins contained in the cytosol are involved in PLD activation. They require the exchange of GDP to GTP for their activation and stimulation of other proteins (76,77). GTPγS actively replaces GDP on the small molecular weight G-proteins for activation in *in vitro* systems. PLD dependence on small G-proteins, including ADP-ribosylation factor (ARF) and RhoA family members, indicate the strong dependency of PLD on GTPγS for activation (56,69). The specific roles of ARF and RhoA on PLD activity will be discussed in sections 1.10a and 1.10b respectively.

PLDs exist in many species and there are two known mammalian phosphatidylcholine-specific isoforms that have been identified, PLD1 and PLD2. PLD1 and PLD2 share approximately 50% sequence homology with each other (78-81). Several regions of conserved sequence have been observed between mammalian and other strains of PLD from bacteria and plants (78,82,83). In worms, flies and

yeast, a single PLD gene appears to be present (84). However, in mammals, upon sequence comparison, PLD genes arose through a duplication of an ancestral gene after divergence occurred between the lower eukaryotes (79,80,85). Both enzymes catalyze the hydrolysis of PC to PA, but they have different requirements for activation. PLD1 is dependent upon small molecular weight G-proteins and GTPγS, while PLD2 is largely independent of these stimuli. PKC can activate PLD, however, activation of different PLD isoforms by specific PKC isoforms has not been completely defined. Both isoforms have an absolute requirement for phosphatidylinositol-4,5-bisphosphate (PIP_2) (56,69). PLD2 also requires increased Ca^{2+} for activation (79,86,87). These differential requirements for activation imply that the two isoforms have differing roles, which can mediate different responses in the cell. Although many of these studies have characterized PLD requirements for activation *in vitro*, regulation *in vivo* appears to involve phosphorylation and translocation of the enzyme (88-90).

1.10 PHOSPHOLIPASE D1

The two isoforms of PLD, PLD1 and PLD2, catalyze the same reaction converting phosphatidylcholine (PC) to phosphatidic acid (PA). However, the two isoforms are differentially regulated and thus, have potentially different biological functions. PLD1 is activated by the small molecular weight G-proteins ARF and Rho, as well as the PKCα and β isoforms. Activation of PLD1 by these factors acts in a synergistic manner (91,92). These effectors which activate PLD1 have been demonstrated to interact directly with the enzyme due to their ability to activate PLD1 in the absence of other proteins in the assay (78,92). The regions held in common between PLDs include CRII, CRIII, and CRIV (93) which are all critical to catalysis *in vitro* and PLD function *in vivo*. Rat PLD1 has two highly conserved regions called HKD regions with one HKD region located in both the amino and carboxy termini (82). The amino and carboxy termini appear to physically interact with each other to

produce catalytic activity (94). The need to determine the distribution of each PLD isoform is essential to the understanding of the signal transduction pathways which are mediated by these PLDs in thyroid cells.

PLD1 has been shown to exist as at least two distinct variants labelled PLD1a and PLD1b, with PLD1a containing 38 additional amino acids in comparison with PLD1b (92). The difference in length of the two proteins most likely involves a differential splicing event. Both of these splice variants have the same catalytic activity and are regulated by the same factors including ARF, RhoA, and the PKCα isoform (92). Both PLD1a and b also appear dependent on Mg^{2+} but are insensitive to Ca^{2+} concentrations (92).

It has been shown previously that PLD1 in HL60 cells can be activated by PKCα, and to a lesser extent PKCβ, in an ATP-dependent manner (95). The amino terminal 325 amino acids are required for PKCα activation of PLD but not for activation by ARF or RhoA (96). However, this region does not contain the sole interaction site for PKCα as determined by deletion mutants and this region may also play a role in inhibiting basal PLD1 activity *in vivo*. In other cell systems, the activation of PLD1 by PKCα acts through a novel mechanism which is ATP-independent and appears to involve PKCα's regulatory domain rather than its catalytic domain (92,97). The interaction site for PIP_2 with PLD1 lies outside the amino terminus domain (78). Mutations in the phox (PX) domain of the amino terminus were unresponsive to PKCα, ARF or Rho *in vitro* (96). This observation is difficult to interpret as the elimination of a single domain leads to the inability to respond to multiple activating factors. However, it was postulated that one consequence of small G-protein binding was to induce a conformational change in the amino terminus to allow catalysis. Without the PX domain, these factors are unable to bind and induce the conformational change which would allow PLD to act.

The sequences specific to PLD1 confer the specific regulatory properties of the enzyme. A region unique to PLD1 referred to as the "loop" region has been shown to mediate the inhibition of PLD1 without stimulation (96) and may serve as an effector regulatory region. PLD1b is missing 33 amino acids from the "loop" region (92). The difference in loop size does not appear to interfere with the regulatory function of PLD1 (92). However, in rats, it has been suggested that there are some subtle changes in response to Rho (85). The amino terminus of PLD1 is conserved between species but it is different from PLD2. In this region there is a weakly conserved PX domain (83) which is involved in a variety of protein-protein interactions (98). There is also a weakly conserved pleckstrin homology (PH) domain (99) which can be frequently involved in mediating inositol phosphate binding (PIP_2). The final 41 amino acids of the carboxy terminus of both PLD1 and PLD2 appear to be involved in catalysis as these sequences are conserved between the two enzymes (83). Modifications at the carboxy terminus, but not at the amino terminus, disrupt enzymatic function (96) confirming this idea.

PLD1 cDNA was the first PLD isoform cloned (78) and its protein domains have recently been determined (96). PLD1 has low basal activity which is sensitive to stimulation by different agonists, including TSH in FRTL-5 thyroid cells (53). This activation of PLD1 by agonist has been shown in several cell types (56,69). PLD1 contains a catalytic domain responsible for causing the removal of the choline moiety from PC producing PA. However, PLD1's "loop" region may have an inhibitory effect on PLD1 binding to PC for hydrolysis. This "loop" region could be moved or altered in some way upon agonist stimulation to allow for PLD1 enzymatic activity to occur. This mechanism has been proposed to demonstrate how PLD1 could be regulated *in vivo* (96). PLD1 is also regulated by other factors including ARF, RhoA and PKCα (56,69) which may, themselves, be activated upon agonist stimulation. The

mechanism of their interactions are being defined, although they have not been clearly established to date.

1.10a ARF

Due to the fact that PLD1 is activated by ARF in many cells, the nature of ARF proteins is of interest to determine their interaction with PLD1 and how they mediate their effects in the cell. ARFs are 20 kDa guanine-nucleotide binding proteins originally purified due to their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A (CTA) subunit. ARFs are part of the Ras superfamily. They are important in vesicular trafficking in almost all eukaryotic cells (76,100). Both the active and inactive forms of ARF are necessary for the proper cycling in vesicular transport. Transition from active to inactive forms is very slow and dependent on the presence of guanine nucleotide exchange factors (GEFs), which cause the switch from the GDP bound to the GTP bound state on small G-proteins, and GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity of the small G-proteins. Myristolation of ARF makes it a better substrate for GEFs (76). GDP dissociation inhibitors (GDIs) have also been identified for both ARF and Rho which inhibit the ability of small G-proteins to dissociate from GDP. ARF is also known to interact with PIP_2 (101), coatomers (102), arfaptin (103), G-protein $\beta\gamma$ subunits (104,105), and $G\alpha_s$ (104). ARF may associate as a dimer or tetramer (102) and the crystal structure of the dimer has been published (106).

Mammalian ARFs are divided into three classes based on their size, amino acid sequence, gene structure, and phylogenetic analysis. ARF 1, 2 and 3 are in class I; ARF 4 and 5 are in class II; and ARF 6 is in class III (107). ARF1 and ARF3 have a role in ER to Golgi transport (100,108). ARF6 is

associated with a pathway involving the plasma membrane and a tubulovesicular compartment distinct from characterized endosomes (109,110). ARFs interact with coatomer proteins and allow budding and formation of vesicles. Studies have also shown that PLD may have a role in vesicle formation (111) and fusion (112) possibly through its connection to ARF. It has been suggested that PLD may be a major regulator of ARF GAPs (113). Inhibition of ARFs by Brefeldin A blocks GEF-catalyzed ARF activation (114,115). These characteristics of ARFs place them in an important position in many cellular processes.

Many research groups have looked at the regulation of ARF and the function that ARF plays in the cell. Several groups have shown that upon activation of cells with agonist, ARF is translocated to the membrane fraction (116). This is consistent with the finding that components from the cytosolic fraction, small G-proteins, are necessary for the full activation of PLD (73-75). As discussed above, ARF plays a role in the activation of PLD1 along with Rho and can act synergistically with Rho to activate PLD1 (56,69). This synergistic activation of PLD1 could establish ARF as a necessary factor for the many cell processes outlined above.

Arfaptin1 is a 39 kDa protein which is involved in Golgi function and is associated with ARF (103). Studies have been done to suggest that arfaptin1 acts as an inhibitor of ARF actions *in vitro*, with the same possibility *in vivo*. A study by Schurmann *et al.* (117) identified an ADP-ribosylation factor-related protein (ARP) which is a membrane associated GTPase with remote similarity to ARFs. The study suggests that the GTP-bound form of ARP binds a specific domain of an ARF GEF and may be involved in a pathway inhibiting ARF-controlled activation of PLD. The role of ARFs in mediating the action of PLD has been studied in many cells and several factors, in addition to the ones

noted above, have been implicated in ARF function. The study of Rho interactions with ARF and PLD1 could provide some insight into ARF's own function.

1.10b RHO

The Rho family consists of three distinct subfamilies Rho, Rac and Cdc42, which control the actin cytoskeleton in different ways (77). There are seven distinct proteins in the family; Rho (A,B,C), Rac (1,2), Cdc42 (Cdc42HS, G25K), RhoD, RhoG, RhoE, and TC10. Rho proteins act as molecular switches to control cellular processes by cycling between the active and inactive states. GDIs appear to stabilize the inactive form while GEFs switch Rho proteins to the active form. Rho signals a number of cellular responses including the reorganization of the actin cytoskeleton and changes in gene transcription (77).

Rho proteins control the actin cytoskeleton in all eukaryotes (77). Activation of Rho in fibroblasts results in the formation of stress fibers and clusters integrins into focal adhesion complexes (118,119). Rac activation promotes *de novo* actin polymerization at the cell periphery (120,121). Cdc42 triggers actin polymerization to form filopodia or microspikes (122). Cross-talk between Rho proteins has been observed (120,121). The Rho family of proteins have been implicated in cell movement, axonal guidance, cytokinesis, and morphogenetic processes involving changes in cell shape and polarity (123,124).

The MAPK cascade has been shown to be activated by the Rho family member Ras (125,126). The MAPK cascade, as well as the JNK/stress-activated protein kinase (SAPK) are known to control gene transcription in response to cellular stresses like UV light or osmotic shock challenge with

inflammatory stimuli (127). Rho family members have also been shown to activate the transcription of serum response factor (SRF) and to activate NFκB (128,129) in response to the formation of reactive oxygen species as Rac activates NADPH oxidase in phagocytes (130). Rho proteins are also required during G1 cell cycle progression due to their actin cytoskeleton and/or gene transcription activities (131-135). From the wide range of activities attributed to Rho family proteins, it is not surprising that PLD is activated by Rho as well.

A large family of Rho GEFs has been identified which share two common motifs, the Dbl homology domain, which may encode the catalytic nucleotide exchange activity, and a PH domain which may determine subcellular localization (123,136). It is not known why there are so many GEFs associated with Rho. In fibroblasts, the α subunit of heterotrimeric G proteins, G_{12} and G_{13}, along with an unknown tyrosine kinase, are required to link the LPA receptor to Rho activation, whereas a phosphatidylinositol-3' kinase (PI3K) is required to link the platelet-derived growth factor (PDGF) receptor to Rac activation (137,138). The location of GEFs in these pathways is unknown. The release of GTPase from GDI is another likely mechanism for Rho activation, which could even be the rate limiting step as GEFs added to the GTPase-GDI complex *in vitro* are unable to activate Rho proteins (77). A recent report suggested that members of the erzin/radixin/moesin (ERM) family of proteins can dissociate GDI from the complex (139). The regulation of Rho is not yet well understood due to the number of factors that are potentially involved.

Rho has been shown to be the target for a serine/threonine kinase, p160Rho kinase, as overexpression of this kinase can induce stress fiber formation independent of Rho (140-142). Substrates for this kinase are the myosin binding subunit of the myosin light chain phosphatase and the myosin

light chain itself (123,143,144), which can lead to the formation of stress fibers. There was a report suggesting that Rho can regulate the production of PIP_2, which is known to have an effect on the actin cytoskeleton (145). PIP_2 plays a role in ERM activation and this may be the mechanism by which Rho functions to maintain PIP_2 levels (146,148). PIP_2 may be essential along with Rac for the release of capping proteins from barbed ends of actin filaments required for actin polymerization in response to thrombin (149).

The role that Rho plays in the activation of PLD has been discussed briefly in section 1.10. In HL60 cells, C3 exoenzyme from *Clostridium botulinum*, an inhibitor of Rho proteins, also inhibited PLD activation by agonist (150). Also, the addition of Rho-GDI inhibited the activation of PLD by GTPγS (151,152). These observations confirm the role that Rho plays in the activation of PLD by agonist. The interaction site between RhoA and PLD occurs in the switch I region of RhoA, which is the common effector domain of Ras-like G-proteins (153). Residues in the switch II region and other internal regions are responsible for the differential activation of PLD by RhoA and Cdc42HS (153). Rho family proteins have been suggested to mediate intracellular signalling from growth factor receptors and heterotrimeric G-proteins to plasma membrane-associated PLD (69,154). The diversity of biological functions related to the activation of Rho proteins is significant, and the role that Rho plays in the activation of PLD is just beginning to be understood.

1.11 PHOSPHOLIPASE D2

In contrast to PLD1, PLD2 has some unique properties which distinguish it from PLD1. PLD2 has an intrinsically high basal activity which does not appear to be stimulated by agonist (79). Thus, PLD2 activity is controlled by repression of the

enzyme. Overexpression of PLD2 appears to affect cytoskeletal structure (79). PLD2 is not activated by the small molecular weight G-proteins ARF and RhoA, or by PKCα *in vitro* (79) which implies an independence of GTPγS for activation. However, there have been some studies done in which PLD2 may be responsive to distinct isoforms of ARF under certain conditions (81). However, wild type PLD2 under normal conditions does not appear to be regulated by the addition of either G-proteins or GTPγS.

The protein domains of PLD2 have also recently been defined (84). The carboxy terminus is similar to that of PLD1 and is involved the in catalytic activity of the enzyme (84,96). The amino terminal 308 amino acids are required for PLD2's high basal activity, specifically amino acids 235-308 (84). Surprisingly, PLD2 lacking this region becomes highly responsive to ARF (84), although PLD2 is not responsive to ARF under normal conditions. This PLD2 deletion mutant may be stimulated by ARF directly or through some other factor, however, the relevance of this observation may be to suggest that *in vivo*, some form of truncation may occur to make PLD2 responsive to ARF. The amino terminus in other proteins has been shown to interact with membranes, but this is not the case for PLD2 (79,93,155). PLD2 was not responsive to PKCα or Rho family members (93). The "loop" region, distinct to PLD1, does not appear in PLD2, which may account for PLD2's high basal activity and low response to agonist. When the "loop" region of PLD1 was inserted into PLD2 and this PLD2 overexpressed, basal PLD2 activity was decreased and became responsive to agonist (84), characteristic of PLD1. In addition to the characterization of PLD2 functional domains, PLD2 splice variants were also identified which result in slightly different length PLD2 proteins, but which have similar function and regulation to each other (99).

Protein function is often characterized by the specific domains which are found within its structure. Phox (PX) domains are characterized as regions involved in

protein-protein interactions (98) while pleckstrin homology (PH) domains act as membrane adapters for signalling proteins via binding of inositol lipids (99). Thus, in PLD2 the PH domains are most likely responsible for the binding of PIP_2 and the further activation of the enzyme.

A study was performed to investigate the link between PLD activation and the MAPK cascade. PLD was activated by insulin and the subsequent accumulation of PA was linked to activation of the MAPK cascade (156). Brefeldin A, which is an inhibitor of the small G-protein ARF, inhibited insulin-mediated PA activation of MAPK (156). Another group showed that stimulation of Rat-1 fibroblasts overexpressing the human insulin receptor (HIRcB cells) activates PLD exclusively through the ARF pathway (157). In contrast, activation of PLD by insulin in adipocytes appears primarily Rho-mediated (158). Thus, the activation of PLD is dependent on the cell type involved and the agonist used to stimulate the PLD activity. The role of PA in these HIRcB cells was studied and PA was shown to recruit Raf-1, a serine/threonine kinase, to the membrane for activation of MAPK with additional stimulation from the small molecular weight G-protein Ras. However, Raf-1 is not activated by PA; PA simply recruits this protein to the membrane where other factors are able to activate it (156). Further evidence that PA interacts directly with Raf-1 *in vitro* was shown by Ghosh *et al.* (159). These observations suggest a role for the product of PLD activity, PA, in biological function.

Rizzo *et al.* attempted to characterize which isoform of PLD was involved in PLD activation by insulin by overexpressing catalytically inactive variants of the two isoforms, PLD1 and PLD2 (156). The use of a catalytically inactive PLD2 variant, but not a PLD1 variant, could block insulin-mediated PLD activation. Thus, they concluded that insulin specifically activated PLD2 in HIRcB cells (156). However, the mechanism by which this mutant downregulates insulin-mediated PLD activity is not clear. Further evidence from these authors suggest that PLD2 may be regulated *in vivo*

by ARF, but these results are also inconclusive (156). ARF1 stimulation of hPLD2 activity in insect cells was also reported to occur at about 2-fold. However PLD1 stimulation was seen at 20-fold (81). In order to determine if the regulation of PLD2 *in vivo* was similar to that *in vitro*, Slaaby *et al.* (90) overexpressed both PLD1 and PLD2 and attempted to determine if they could stimulate PLD2 with epidermal growth factor (EGF). They showed that PLD2 was activated to a small extent by EGF, although PLD1 was activated to a much higher extent. They showed that PLD2, but not PLD1, associates with the EGF receptor in a ligand-independent manner and becomes tyrosine phosphorylated upon EGF receptor activation. This was the first study to demonstrate how each PLD isoform interacts with the EGF receptor *in vivo* and also that agonist-induced tyrosine phosphorylation plays a role in PLD2 regulation. Further study by this group demonstrated that an EGF receptor kinase is essential for stimulation of PLD2 activity, while tyrosine phosphorylation of PLD2 is not. Thus, it must have a different mode of interaction. Despite these findings, the regulation of PLD2 has been difficult to study because of its high constitutive activity and the difficulty in measuring PLD1 versus PLD2 activity both *in vitro* and *in vivo*. The investigation into tyrosine phosphorylation of PLD2 provides a possible mechanism of regulation for PLD2, as well as PLD1.

1.11a TYROSINE PHOSPHORYLATION

Protein tyrosine phosphorylation comprises about 0.1% of total cellular protein phosphorylation but plays a crucial role in growth, differentiation, and cell metabolism (160). Tyrosine kinases are divided into two classes, the receptor and non-receptor (cytosolic) types. The receptor type tyrosine kinases are typically activated by growth factors and activation of the intrinsic activity of the receptor (161,162). The non-receptor tyrosine kinases may be involved in a similar mechanism but other factors mediate the events (162). The role of

tyrosine phosphorylation of PLD has not been well characterized. There are at least four possible mechanisms by which PLD is regulated including regulation through PKC, Ca^{2+}, small G-proteins, and tyrosine phosphorylation (160). Several studies have been done to show that tyrosine phosphorylation is involved in the activation of PLD either by phosphorylation of PLD itself, or through the intrinsic tyrosine phosphorylation of certain growth factor receptors (163-166). The involvement of tyrosine kinases in PLD activation is demonstrated through the use of tyrosine kinase inhibitors, like genistein and tyrphostin, which block PLD activation (163,167). The use of tyrosine phosphatase inhibitors, like vanadate or pervanadate, which block the removal of tyrosine phosphorylation, increase PLD activation (168,169). H_2O_2, vanadate, and pervanadate increased tyrosine phosphorylation in several proteins in endothelial cells (170) and neutrophils (171). Further studies showed that H_2O_2 plus vanadate induced tyrosine phosphorylation of PLD itself (172) and this effect was dependent on interactions with PKCα. In neutrophils, fMLP caused an increase in tyrosine phosphorylation and PLD activation (168). fMLP is also known to increase the respiratory burst through NADPH oxidase and this may be responsible for increased tyrosine phosphorylation and PLD activity (160).

In addition to the involvement of tyrosine kinases in PLD activation, there appears to be some interaction of small G-proteins mediating the effects. Agonists which are coupled to G-proteins stimulate tyrosine kinase dependent phosphorylation of certain cytosolic and membrane proteins (173-175), which could include PLD. In neutrophils, fMLP stimulation of PLD was sensitive to pertussis toxin (176), a G-protein inhibitor. Tyrosine kinase involvement in signalling upstream and downstream of Rho activity was also observed

(118,120,177). Based on this and other evidence, the involvement of G-proteins in tyrosine phosphorylation and activation of PLD was postulated.

1.12 PHOSPHATIDIC ACID

The product of PLD activity, PA, is a known intracellular signalling molecule with diverse cellular activities. PA itself is a short lived molecule derived not only from PLD action on PC, but also from other sources in the cell (178). Some biological functions associated with PA are decreasing adenylyl cyclase in fibroblasts (57), increasing superoxide formation and degranulation in neutrophils (58,59), stimulating MAPK (60), and activating serine/threonine kinases (179,180). Evidence for the involvement of PA in superoxide formation and NADPH oxidase activation comes from studies done which show that a decrease in PA production from PLD diverted by ethanol to produce phosphatidylethanol, also caused a decrease in the activation of NADPH oxidase (181,182). Increases in PA concentrations with the addition of DL-propranolol, which blocks PA conversion to DAG, caused an increase in the activation of NADPH oxidase (183). Recent studies have shown that PA can directly activate the PKCζ isoform (184). PI3K which produces PIP_3 can also activate this PKC isoform (185,186). In regards to thyroid function, PA was found to increase the production of thyroid hormones (52,187). PA is known to stimulate a respiratory burst resulting in H_2O_2 production (58,59). As discussed in section 1.3, this is an essential component of iodide organification which is a major step in thyroid hormone synthesis (6). Thus, PA may be a critical component in thyroid cell biology.

PA itself may not be the only component involved in mediating the effects of TSH in thyroid cells. PA is rapidly converted to diacylglycerol (DAG) through the actions of LPP (188). DAG is another known signalling molecule, via PKC activation, with a diverse range of effects in the cell. Although the exact role of PA has yet to be

determined in the thyroid cell, the importance of PA to cell signalling has been established.

1.13 DIACYLGLYCEROL

Diacylglycerol is a lipid which plays a crucial role in lipid biosynthesis (64). In addition to this role, DAG has been identified as a physiological activator of conventional and novel PKC isoforms. DAG's role in PKC activation is essential in many signal transduction pathways producing a wide variety of cellular responses. DAG binds to the cysteine-rich regions of PKC and activates the enzyme in concert with other factors including phosphatidylserine (PS) and Ca^{2+} (64). This thesis will focus on DAG as a signalling molecule rather than its role in lipid biosynthesis. DAG itself can be produced from a number of different pathways and is found both downstream and upstream of PLD activation. There are two phases of DAG production upon agonist stimulation; a first phase occurring within two minutes of exposure, and a second phase occurring much later. Upon TSH stimulation, phospholipase C (PLC) is activated and hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to DAG and inositol 1,4,5-trisphosphate (IP_3) (189,190). As stated above, DAG is an activator of certain PKC isoforms, while IP_3 increases intracellular Ca^{2+} levels. This represents the first phase of DAG production. Downstream of PLD activation, there is an accumulation of PA as a consequence of the actions of PLD on PC. The conversion of PA to DAG represents the second phase of DAG production derived from PLD and PC. This biphasic production of DAG has been observed in many cell types aside from FRTL-5 thyroid cells (191,192), and places DAG in a very dynamic position in cell signalling with the possibility of activating different PKC isoforms at different time points after agonist stimulation.

The two phases of DAG production stem from different enzymes. The first phase of DAG is derived from PLC and phosphoinositides while the second phase is

derived from PLD and PA. Since there are approximately 50 different mammalian DAG species identified to date, characterized by their fatty acid composition (64), it would be logical to suggest that different sources of DAG lead to different DAG species. Bocckino *et al.* demonstrated that, by the analysis of DAG fatty acid composition in stimulated cells, certain DAG species could be attributed to the hydrolysis of PC rather than phosphoinositides (191). Consequently, with the production of different DAG species, the activation of different PKC isoforms becomes a possibility. In effect, the presence of different DAG and PKC isoform activation could lead to different biological responses in the cell. In this capacity, DAG not only generally plays a role in the activation of PKC, but, depending on the fatty acid chains present, may activate specific PKC isoforms preferentially.

Studies have shown that early after agonist stimulation, the DAG produced is mostly of the polyunsaturated form (193,194). This is consistent with PIP_2 hydrolysis. The sustained levels of DAG are predominantly monounsaturated or saturated (195,196) which is consistent with the products of PLD activity. The more polyunsaturated DAGs appear to be better activators of PKC (197). This would make early phase DAG from PIP_2-PLC the better activators of PKC. There is also some suggestion that the monounsaturated and saturated DAGs may not be able to activate PKC (195). It is not entirely clear how the different DAG species are able to differentially activate PKC isoforms, but the results of these and other studies suggest that polyunsaturated DAGs and monunsaturated and saturated PAs act as the signalling molecules in the cell (64).

Although the focus of this thesis is in regards to PLD, it is not the only source of second-phase DAG in cells. There is a PLC activity capable of converting PC directly to DAG releasing phosphorylcholine. However, there is evidence that this source of DAG does not play a major role in regulating DAG concentrations in agonist-stimulated cells (64) as it appears to have a greater role in sphingomyelin synthesis

(198). However, it cannot be completely eliminated as a source of DAG in several cell types as studies have shown that GTPγS and purinergic receptor stimulation generate both choline and phosphorylcholine, the products of PLD and PLC respectively (199).

Evidence for the generation of DAG through the agonist-stimulated PC-PLD pathway is substantial in comparison to that generated from PI-PLC. The product of PLD activation, PA, is converted to DAG by LPP which appears to be solely regulated by substrate concentration (70). PC-PLD activity is stimulated by the same agonists that activate PI-PLC. However, the PLD activity remains observable after the PLC activity has been desensitized (200,201). This suggests that PC-PLD is responsible for the sustained levels of DAG in the cells. DAG is important in the activation of PLD due to its interaction with and activation of PKC. The two-phase accumulation of different DAG species may be the key to understanding the regulation of thyroid hormone synthesis through PLD. Since different DAG species may activate specific PKC isoforms, these isoforms have the potential to mediate different cellular activities.

1.14 PROTEIN KINASE C

PKC comprises a large family of serine/threonine kinases which are activated by many intracellular signals. PKC has been shown to be regulated by phosphorylation as well as lipid second messengers including DAG and PS (72). PKCs have been implicated in a wide range of signal transduction pathways and physiological processes in the cell including proliferation, apoptosis, platelet activation, remodeling the actin cytoskeleton, and modulation of ion channels (72).

The mammalian PKC enzyme family consists of at least 12 distinct protein kinases. These enzymes are divided into three subgroups based on their structural properties and cofactor requirements. The conventional isoforms (α, βI, βII, and γ) require phosphatidylserine (PS) for activity and are stimulated by Ca^{2+}, DAG, and phorbol esters (202-206). The novel isoforms (δ, ε, η, θ, and μ) also require PS and

are activated by DAG, but not by Ca^{2+} (207-214). The atypical isoforms (ζ, λ, and ι) are not activated by DAG, Ca^{2+}, or phorbol ester (207,215,216) but do respond to PS . In addition to the differential regulation, each subgroup exhibits distinct tissue expression and subcellular distribution (217-219). The conventional PKC isoforms translocate to the membrane fraction after stimulation with DAG or phorbol ester (220-227). Association of PKC with the membrane fraction is often required for its initial activation but it also enhances the rate of its subsequent proteolytic degradation (72). This results in the downregulation of PKC with prolonged treatment with phorbol esters which virtually eliminates PKC activity (228-231). It has also been shown that different PKC isoforms are downregulated at different rates and by different substrates (232-236). These differences in regulation and expression are consistent with the concept that different isoforms mediate different cellular functions which has been established in some cells (72).

In 1981, the first observations that activation of PKC leads to PLD activation and choline production were made (237). The PKCα and PKCβ isoforms are capable of activating PLD (238-240), while in most cells, other PKC isoforms are unable to do so. A recent paper by Hodgkin *et al.* (241) demonstrated that PKCδ was also able to activate PLD to levels similar to that produced by PKCα in HL60 cells. This group suggested that the novel isoforms have similar domains to the conventional isoforms, although the arrangement is different. This property may allow the novel forms to activate PLD under certain conditions. A hormone activator of PLD, platelet-derived growth factor (PDGF), is dependent on the activation of PLC and the formation of DAG (242-245) and Ca^{2+} for PLD activation. G-proteins also mediate the effects of PKC on PLD activation (72). These observations contribute to the complexity by which PLD is regulated and demonstrate the possibility that PLD could be involved in a wide variety of physiological processes.

The role of PKCs in PLD activation is an important aspect of regulation in many cell types, including thyroid cells. In particular, the involvement of different PKC isoforms will play a critical role in understanding how specific pathways are regulated. In reference to the proposed pathway of TSH action in FRTL-5 thyroid cells presented in section 1.8, the presence of two separate DAG and PKC phases focuses on the idea that different sources of DAG lead to differential activation of PKC isoforms which can then have different biological roles in the cell. There is evidence of differential regulation of the PKC isoforms in recent years. The myristolated alanine-rich C-kinase substrate (MARCKS) protein, which is a substrate for PKC in all cells, is phosphorylated by all PKCs except PKCζ (246). The heterogeneous ribonucleo-protein A1 (hnRNP A1) is phosphorylated by PKCζ, but not other PKCs (247), while elongation factor eEF-1α is a good PKCδ substrate , but not for other PKCs (248). PKCε has been suggested to be an oncogene modulating the MAPK pathway (249-251), although some conflicting evidence exists (252). Over the past several years, there has been evidence to suggest that PKCζ and PKCλ are mediators of mitogenic signal transduction (253,254). Further studies with PKCζ show that it was strongly activated by PA in the absence of Ca^{2+} , while PKCα was activated by PA but only in the presence of Ca^{2+} (184). PA physically binds to PKCζ and these results suggest that PA may be a physiological activator of PKCζ. The results from another study show that PKCβ has a critical role in the mediation of PLA_2 activation by PMA, while PMA uses PKCε and/or μ to up-regulate adenylyl cyclase activity (255). These observations demonstrate how different PKC isoforms have different substrates and targets, which implies that their physiological roles inside the cell are different. FRTL-5 thyroid cells contain the PKCα, δ, ε and ζ isoforms which provide representatives of each class of PKC in these cells (256). Thus, the role of these isoforms in thyroid function will be critical to understanding how PKCs interact in the thyroid gland.

1.15 LYSOPHOSPHATIDIC ACID

To further understand the complexity of PLD activity in thyroid cells, another signalling molecule, lysophosphatidic acid, is of interest. Lysophosphatidic acid (LPA; 1-acyl-*sn* -glycerol-3-phosphate) is an extracellular phospholipid mediator of many signalling pathways (61). The generation of PA in cells is rapidly converted to DAG or, through the actions of phospholipase A_2 (257), is converted to LPA. Its signalling properties are currently being studied, but the role that LPA plays in signal transduction and biological function has not been clearly established. LPA may act intracellularly, but most studies focus on the extracellular role of LPA as it is secreted by some cells (61). LPA binds to external membrane, G-protein coupled receptors which are present in many cell types (258). It is found in serum in an albumin-bound state (258). LPA has been shown to be involved in a number of biological functions in different cells including cell proliferation, platelet aggregation, smooth muscle contraction, stress fiber formation, and neurotransmitter release (61-63). LPA is produced and secreted from cells including activated platelets (258) which implicates a role for LPA in blood clotting. The addition of exogenous LPA to cells leads to an increase in intracellular Ca^{2+} concentration in several cell types (258). This Ca^{2+} mobilization induced by LPA acts through both a pertussis toxin-sensitive (G_i) and -insensitive (G_q - activate PLC) mechanism (259). This list continues to grow as new and diverse roles for LPA are established in different cell types. The significance of LPA as a signalling molecule is slowly becoming clearer as new techniques and discoveries allow us to define the exact role and mechanism by which LPA acts.

The search for the LPA receptor allowed greater insight into the mechanism by which LPA could interact with cells and elicit a specific response. Recently, the cDNA for the LPA receptor has been cloned. One group, lead by Hecht (260), discovered a gene they labeled *ventricular zone gene-1* (*vzg-1*), which was highly expressed in a specific mouse cell line. This gene was consistent with the description for a specific

membrane protein of 39-40 kDa that met the criteria for the LPA receptor as defined by Van der Bend *et al.* (261). The sequence cloned by Hecht *et al.* had almost 96% homology to the gene sequence of sheep orphan GPCR cDNA labeled *endothelial differentiation gene -2* (*edg-2*) (262). This same gene was also cloned by Macrae *et al.* (263) who termed it *rec1.3* and demonstrated its wide distribution in mouse tissues. Erickson *et al.* (264) confirmed that the human *edg-2* homologue was a functional LPA receptor after overexpressing this gene in yeast lacking an endogenous LPA receptor. The *edg-2* receptor coupled to G_i was shown to be involved in the inhibition of adenylyl cyclase (260,265), as well as leading to a PLC-mediated Ca^{2+} increase in Jurkat and HTC4 cells. *Edg-2* also conferred LPA-induced stress fiber formation and neurite retraction in a Rho-dependent manner consistent with the *edg-2* association with $G_{12/13}$ (266). This *vzg-1/rec1.3/edg-2* receptor shares some homology (35%) with a G_i -linked receptor termed *edg-1*. The *edg-1* receptor has been shown to be bound by LPA and induce the activation of mitogen-activated protein kinase (MAPK) (267). Another group, Tigyi *et al.* (268), isolated another putative LPA receptor from *Xenopus* oocytes. Sequence homology between this receptor and *vzg-1* is unexpectedly small, although both receptors appear to have properties of an LPA receptor as the overexpression of this receptor in oocytes affects Cl^- concentrations in response to LPA, but not other lipids. Other *edg* receptors have been cloned and their specific functions in cells are currently being studied. However, several studies indicate that the *edg* receptors involve coupling to some form of G-protein to elicit their response (258). This is an interesting finding and will help to identify the signal transduction pathways for specific cells based on the *edg* receptor present and the G protein that it is coupled to. This would provide a mechanism defining how these receptors have tissue-specific expression and regulation.

The LPA receptor has been shown to couple to at least three distinct G proteins including G_q, G_i, and $G_{12/13}$. Each of these G proteins is linked to different biological

functions in the cell. G_q links the LPA receptor to phospholipase C activation which is involved in the conversion of PC to DAG (258). G_i links the receptor to Ras-GTP accumulation and inhibition of adenylyl cyclase (258). $G_{12/13}$ mediates Rho activation through the LPA receptor. Although these functions are not exclusive and there may be some overlap between receptors, the use of different G-protein coupling to the receptor allows for specific responses in different cell types to ensure specificity of action.

LPA is an agonist that binds its G_i-coupled receptor and stimulates Ras-GTP accumulation and activates a protein tyrosine kinase (269). G_i-coupled receptors are pertussis toxin sensitive (258). One group found that LPA-induced cell proliferation was pertussis toxin sensitive suggesting G_i involvement (259). Accumulation of Ras-GTP places LPA with a role in the activation of MAPK which is known to be involved in cell proliferation (270,271). Thus, LPA has some properties of epidermal growth factor (EGF) which can also activate MAPK. Studies indicate that the $G_i\beta\gamma$ dimer is responsible for Ras-MAPK activation by LPA, and this activation may involve interaction with protein tyrosine kinases (272-274). The role of tyrosine kinases in LPA-mediated MAPK activation is an interesting one. Studies by Daub *et al.* (275) found that LPA increased tyrosine phosphorylation of the EGF receptor. This tyrosine phosphorylation leads to the recruitment of other factors involved in cell proliferation and growth and to Ras-MAPK activation (274). However, other studies do not appear to support this idea (269,276) and further work is necessary to link tyrosine phosphorylation to Ras activation by G_i-coupled LPA receptors. Since EGF and PDGF receptors are tyrosine phosphorylated for activation, LPA appears to have an indirect stimulatory effect on these receptors (275). Upon LPA activation, the $G_i\alpha$ subunit appears to be involved in the inhibition of adenylyl cyclase (277). However, these pathways must be elucidated further.

In conjunction with the activation of the G_i-Ras pathway and tyrosine phosphorylation by LPA, LPA also appears to be involved in activating a

phosphoinositide 3-kinase (PI3K) activity (258). Inhibitors of PI3K blocked the stimulatory effect of LPA on MAPK activation (278). Both the α and γ isoforms of PI3K have been implicated in Ras-MAPK activation (278,279). However, to link G_i to the Ras-MAPK cascade, the γ isoform is favoured (279). The product of PI3K, phosphatidylinositol-1,4,5-trisphosphate (PIP_3), can bind to domains of various signalling proteins including tyrosine kinases which may help to define the role of PI3K in this pathway.

The LPA receptor coupled to $G_{12/13}$ has been implicated in linking LPA to Rho activation in fibroblasts (258). Rho is a family of small molecular weight G-proteins involved in cell signalling in pathways involving actin stress fiber formation, cell rounding, neurite retraction, and gene transcription (77). Downstream signalling of Rho has been the focus of much research and the identification of a Rho-associated serine/threonine protein kinase which is responsive to LPA may help to characterize the role Rho has in mediating the activation of specific signalling pathways by LPA (280). Others activities downstream of Rho include PLD activation, PI3K activation, as well as the activation of several other factors (77). As with LPA induction of G_i proteins and the cell proliferation that follows, the full induction of cell proliferation requires the activation of the $G_{12/13}$ -activated pathway as well.

A new role for LPA in fibroblasts was discovered by Postma *et al.* (281). They found that serum was able to stimulate a dramatic depolarization in the plasma membrane. The serum activity was due to the presence of LPA bound to albumin and the depolarization could be attributed to activation of a Cl^- channel. This Cl^- efflux channel is responsible for creating a membrane potential which consistently follows PLC activation and coincides with the actin cytoskeleton remodelling by Rho (282). The role that LPA plays here must be examined further.

LPA also appears to have a role in inhibiting gap junctional communication (283). Gap and tight junctions are responsible for cell-cell communication. This

communication appears to be regulated by LPA, which inhibits the cell-cell communication (283). It is not surprising that LPA should have a role here, since LPA has been implicated in the suppression of apoptosis induced by antibodies to the T cell receptor (284). Since LPA can be secreted from cells, its ability to act in an autocrine or paracrine fashion lends support to a role in cell-cell communication.

The reason for studying the effects of LPA in FRTL-5 thyroid cells stems from the discovery that LPA is an activator of PLD (61). This was an interesting observation in light of the possible involvement of PLD in mediating the actions of TSH in FRTL-5 thyroid cells. Another interesting note is that TSH has been shown to activate adenylyl cyclase in FRTL-5 thyroid cells and that this pathway leads to the activation of PLD. However, LPA causes an inhibition of adenylyl cyclase (61). Thus, if LPA is still able to activate PLD, it must be acting through a different pathway. Further studies noted that LPA increased PLD activity in a pertussis toxin sensitive manner which implicates G_i protein involvement (61). The response to LPA was also found to be inhibited by C. botulinum toxin (118) which is an inhibitor of the small G-protein Rho. This could indicate the involvement of LPA and Rho on stress fiber formation (77). The effects of LPA on PLD activation in thyroid cells could help give some indication of intracellular components involved in each of the PKA and PKC pathways and provide answers to how PLD mediates the production and inhibition of thyroid hormone synthesis.

1.16 INTRODUCTION TO EXPERIMENTS

In order to understand the intracellular events within FRTL-5 thyroid cells, our lab attempted to identify those intracellular components mediating TSH stimulation. One major factor, as outlined in the introduction, is PLD. Due to the apparent central position of PLD in the pathway mediating the effects of TSH (see section 1.9), its regulation was studied in response to TSH and other external stimuli. Thus, the regulation of PLD in FRTL-5 thyroid cells will help to elucidate the mechanism of TSH action and the regulation of thyroid hormone synthesis.

2. MATERIALS AND METHODS

2.1 MATERIALS

FRTL-5 thyroid cells were kindly provided by Dr. Leonard Kohn from the Interthyroid Research Foundation (Baltimore, MD; patent no. 4,609,622 and 4,608,341). They are continuous functional epithelial cells derived from normal rat thyroid, as originally described by Ambesi-Impiombato *et al.* (7). ARF antibodies were a kind gift of Dr. Sylvain Bourgoin (Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche de CHUL, Québec). Coon's modified Ham's F-12 medium, human transferrin, bovine insulin, cortisol, somatostatin, glycyl-L-histidyl-L-lysine, fatty acid free-bovine serum albumin (BSA), Triton X-100, bovine TSH, L-α lysophosphatidic acid (LPA), dideoxyadenosine (DDA) and sodium tetraphenylborate were obtained from Sigma Chemical Co. (St. Louis, MO). Hank's balanced salt solution (HBSS), newborn calf serum (heat inactivated), and MEM-non essential amino acid solution were purchased from GIBCO-BRL (Grand Island, NY). RhoA antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). PKC antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Phosphatidylethanolamine and phosphatidylcholine were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Phosphatidylinositol 4,5-bisphosphate (PIP_2) and chelerythrine were obtained from Calbiochem (La Jolla, CA). ECL-Plus reagents, [^{3}H]myristate and L-3-phosphatidyl[N-methyl- ^{3}H]choline were obtained from Amersham Life Science (Arlington Heights, IL). GTPγS and GDPβS were obtained from Boehringer Mannheim, Canada. All other chemicals and solvents were obtained from commercial sources and were of analytical grade.

2.2 CELL CULTURE PROTOCOL

2.2a FRTL-5 THYROID CELLS

FRTL-5 thyroid cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% newborn calf serum and six hormone mixture (6H) comprised of bovine insulin (10 μg/ml), cortisol (10 nM), human transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 μg/ml), somatostatin (10 μg/ml), and TSH (10 mU/ml). Cells were maintained at 37°C in an atmosphere saturated with water and containing 95% air-5% CO_2. When confluence was reached, cells were trypsinized and subcultured in 100 mm tissue culture dishes. After the cells approached confluence, the medium was changed to 6H medium devoid of TSH (termed 5H medium), and the cells were incubated for a further seven days. The culture medium was changed twice weekly. On the day of the experiment, the medium was replaced with Coon's modified Ham's F-12 medium containing 0.1% bovine serum albumin (BSA), fatty acid free, without any additional hormones.

2.3 ASSAY PROTOCOLS

2.3a PROTEIN CONCENTRATION

Protein concentrations were measured by the Bio-Rad assay method. The samples were diluted 1:10, 1:25, 1:50, and 1:100. Twenty µl of sample was transferred to individual wells on a 96 well micro-titre plate. To each well, 200 µl of a diluted 1:5 Bio-Rad dye reagent concentrate to water was added and the mixture was left standing at room temperature for five minutes to ensure colour development. Bovine serum albumin (BSA) at 0.1 mg/ml was used as a standard and diluted to generate a concentration curve. Fig. 1 illustrates a BSA concentration curve obtained and this is representative of BSA curves obtained from all other assays. The change of absorbance at 595 nm was detected using a SLT Labinstruments plate reader.

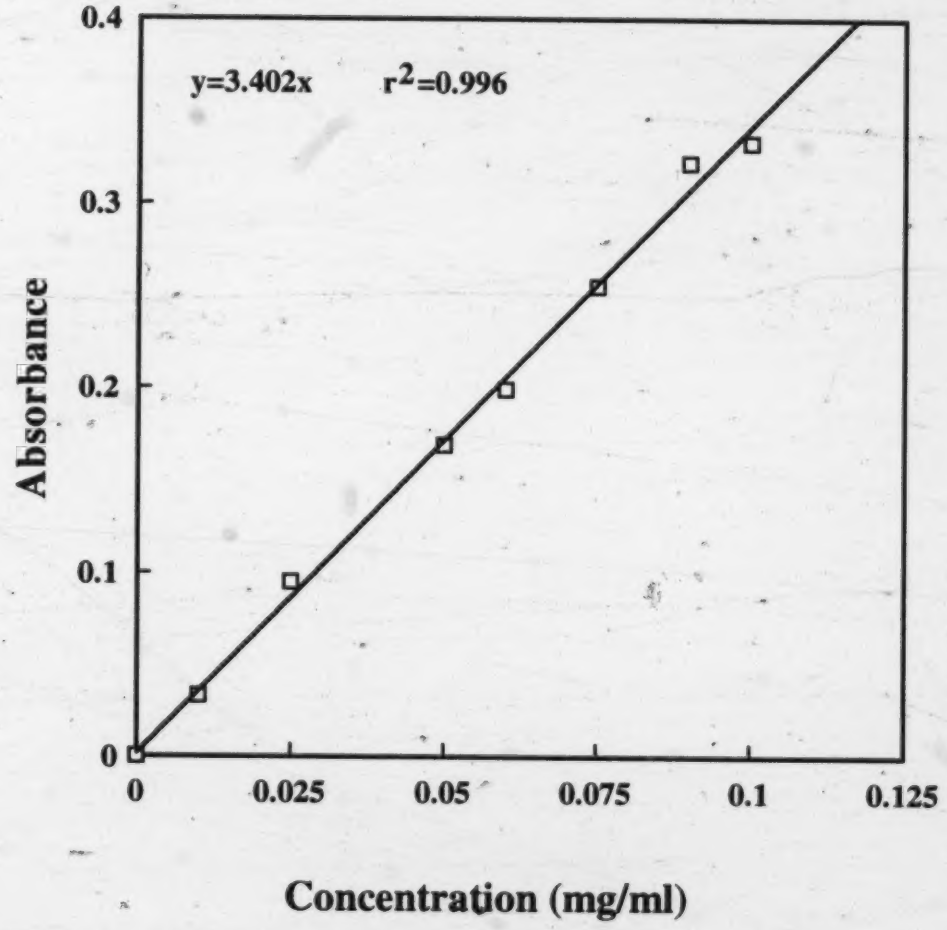
2.3b PHOSPHOLIPASE D AND THE TRANSPHOSPHATIDYLATION REACTION

PLD hydrolyzes phosphatidylcholine to phosphatidic acid and water-soluble choline (56). However, in the presence of a primary alcohol, PLD catalyzes a transphosphatidylation reaction where the alcohol serves as the acceptor for the phosphatidyl moiety (56). FRTL-5 thyroid cells were grown on 60 mm tissue culture dishes at a density of 1.5×10^6 cells per dish to confluence and then incubated in 5H medium for seven days. On day seven, cells were washed three times with Coon's modified Ham's F-12 medium and then incubated in serum-free modified Ham's F-12 medium containing 0.1% BSA and 1 µCi [^{3}H]myristate/ml for 2 h. The reaction media was then aspirated and the cells were washed twice with nonradioactive Ham's medium-BSA solution. The FRTL-5 thyroid cells were then incubated for a further 2 h

FIGURE 1

BSA concentration curve for determination of unknown protein content

Bovine serum albumin (BSA) at 0.1 mg/ml was used as a standard and diluted with water to generate a concentration curve for determination of the protein content of membrane and cytosolic fractions. To each well, 200 µl of a diuted 1:5 Bio-Rad dye reagent concentrate to water was added and the mixture was left standing at room temperature for 5 min. The change of absorbance at 595 nm was detected using a SLT Labinstruments plate reader.


y=3.402x
r2=0.996
Absorbance
0.4
0.3
0.2
0.1
0
0
0.025
0.05
0.075
0.1
0.125
Concentration (mg/ml)

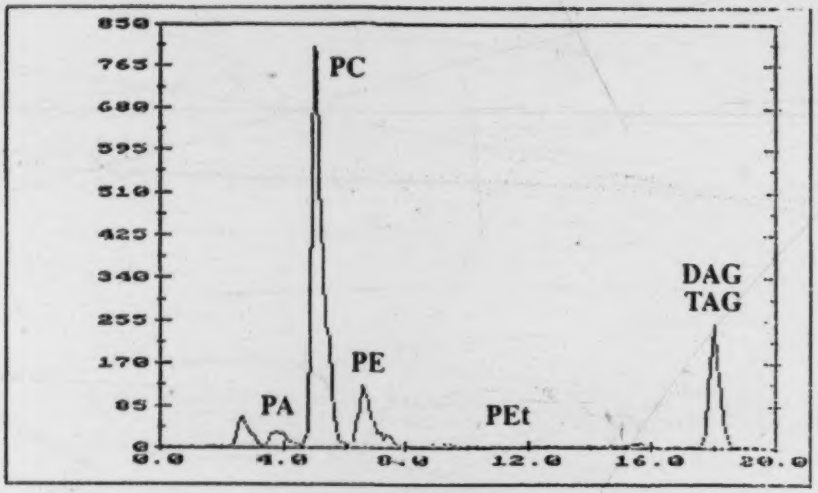
in medium containing 0.5% BSA with three changes of medium. This procedure removes unesterified myristate, thus stopping further labeling but allowing the turnover of prelabelled phospholipids (75% of which was phosphatidylcholine). The medium was then replaced with medium containing 0.1% BSA and 200 mM ethanol in which cells were incubated for five min prior to the addition of test reagents. Phorbol myristate acetate (PMA - 100 nM), lysophosphatidic acid (LPA - 10 and 50 μM), TSH (30 and 100 μU/ml), and hCG (1000 and 3333 U/ml) were added after the five min incubation period and the cells were incubated for a further 30 min as described previously (53,55). The cells were washed twice with ice-cold PBS and scraped in 0.5 ml methanol. The dishes were washed with a further 0.5 ml methanol, and the lipids were extracted from the combined methanol washes using chloroform / methanol / water in a ratio of 1 / 1 / 0.9, with the water containing H_3PO_4 (0.2 M) and KCl (2 M). Lipids were analyzed by thin layer chromatography (TLC) (285) on plastic-backed silica gel plates. For TLC, samples were loaded onto 20 x 20 cm silica gel plates at the 4 cm mark from the bottom of the plate. Plates were run first in chloroform / methanol / ammonia (65:35:7.5) to the 16 cm mark, which allows the movement of all lipids except for PA which remains at the origin. Plates were removed, allowed to dry and run in a second TLC chamber containing petroleum ether / diethyl ether / acetic acid (60:40:1), which separates neutral lipids from other lipids which remain in the same position on the plate. In some cases, the bottom portion of the plate containing PA, LPA and BisPA was cut from the rest of the plate and run in a separate TLC chamber, in the reverse direction, containing chloroform / acetone/ acetic acid / methanol / water (50:20:15:10:5) which allows for the separation of PA from LPA and BisPA. Radioactive lipids were detected with a Bioscan System 200 Imaging Scanner (Bioscan, Inc., Washington, DC). The separation of lipids obtained is shown in Fig. 2. PC makes up about 70-75% of total membrane phospholipid. Other lipids represented are in proportion to PC. PEt is not typically found in cells under normal

FIGURE 2

The separation of lipids on TLC plates before and after PMA treatment

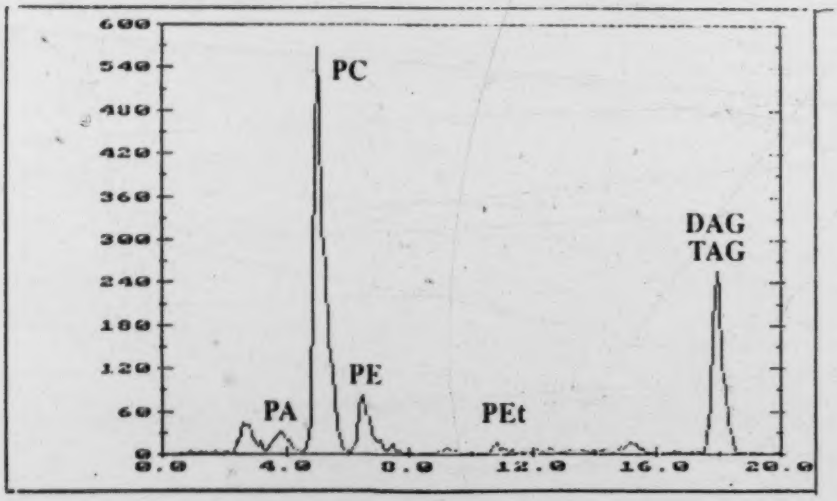
Samples from FRTL-5 thyroid cells scraped from dishes after treatment with test reagents were loaded onto 20 x 20 cm silica gel TLC plates at the 4 cm mark. TLC plates were run in the first phase (chloroform/methanol/ammonia, 65:35:7.5) to the 16 cm mark, removed and dried. TLC plates were then run in a second system (petroleum ether/diethyl ether/acetic acid, 60:40:1) to the top of the plate. The PC peak represents about 70-75% of total cell lipid. Under normal conditions, PEt is not present in cells. Thus, the appearance of a PEt peak in cells treated with PMA in the presence of ethanol represents a significant increase in PLD activity in FRTL-5 thyroid cells. Radioactive lipids were detected with a Bioscan System 200 Imaging Scanner.

a)



Control Plate

b)



+ PMA

conditions as it is a product of the transphosphatidylation reaction. Thus, although the PEt band seen with the addition of PMA is quite small, the appearance of a peak at all represents a significant increase in terms of PLD activity. PEt and PC bands were cut from silica gel plates and quantitated by liquid scintillation counting using EcoLite scintillation fluid with the addition of 1 ml water and 1 ml methanol to each tube prior to the addition of scintillant to ensure the release of the radiolabelled lipid from the silica gel into the scintillation fluid. Agonist-stimulated PEt formation was calculated after subtraction of background radioactivity that was obtained from incubations in the absence of ethanol.

2.3c TREATMENT OF FRTL-5 THYROID CELLS FOR *IN VITRO* PLD ASSAYS

FRTL-5 thyroid cells were plated on 100 mm tissue culture dishes at a density of 2.5 x 10^6 cells per dish. Cells were grown to confluence and incubated in 5H medium for seven days. At day seven, cells were washed twice with Coon's modified Ham's F-12 medium. Cells were then incubated in Coon's modified Ham's F-12 medium containing 0.1% BSA and the test reagent(s) for the times and concentrations indicated. Test reagents used include TSH (10-1000 μU/ml), PMA (100 nM), forskolin (1 nM), and norepinephrine (5 μM), dideoxyadenosine (5 nM) and chelerythrine (1 μM). Incubation times tested include 5, 10, 15, 20 and 30 min.

2.3d ISOLATION OF MEMBRANE AND CYTOSOLIC FRACTIONS FOR *IN VITRO* PLD ASSAYS

Cells treated with agonists, inhibitors or control cells were washed twice with ice-cold PBS and harvested from dishes using Solution A containing NaCl (137 mM), Na_2HPO_4 (8.1 mM), KCl (2.7 mM), KH_2PO_4 (1.5 mM), EDTA (2.5 mM), PMSF (0.1 mM), DTT (1 mM), benzamidine (1 mM), leupeptin (2.5 μg/ml), and aprotinin (2.5 μg/ml) at pH 7.2. Cells were then sonicated using a probe sonicator for 5-10 sec until homogeneity. Total cell homogenate was centrifuged at low speed for 5 min at 4°C and the cellular debris (pellet) was discarded. The membrane fraction was separated from the cytosolic fraction by high speed centrifugation at 99,000 rpm for 1 h at 4°C. Cytosol was removed and membranes were rinsed twice with ice-cold PBS and resuspended in Solution A. Membranes and cytosol were used to assay PLD or for Western blotting experiments.

2.3e PREPARATION OF LIPOSOMES

Liposomes for the in vitro PLD assay were prepared according to Brown *et al.* (286). In summary, the liposome containing PIP_2 (a suboptimum concentration of 2 μM except where stated), PE (138 μM), and [^{3}H]PC (8.6 μM at about 100,000 dpm/assay) was added to each tube in the assay. The lipids were dried down under nitrogen in a glass tube and resuspended in sonication buffer containing HEPES (125 mM), EGTA (7.5 mM), KCl (200 mM), and dithiothreitol (DTT - 2.5 mM). This mixture was sonicated in a bath sonicator for 15 min, put on ice for 30 min, then sonicated for a further 15 min before addition to the incubation system.

2.3f PREPARATION OF MIXED MICELLES

Mixed micelles for the Triton assay were prepared containing PIP_2 (1 μM), [^{3}H]PC (100 μM and about 100,000 dpm/assay), and Triton X-100 (0.1%). The lipids were dried down under nitrogen in a glass tube, Triton X-100 was then added, and the mixture was resuspended in sonication buffer containing HEPES (125 mM), EGTA (7.5 mM), KCl (200 mM) and DTT (2.5 mM). This mixture was sonicated in a bath sonicator for 15 min, put on ice for 30 min, then sonicated for a further 15 min before addition to the incubation system.

2.3g CELL-FREE ASSAY FOR PLD

A second method for the detection of PLD activity using an *in vitro* activity assay was performed. Unlike the transphosphatidylation reaction method, this assay employs the use of choline-labelled [^{3}H]PC. Instead of adding ethanol to the system, L-3-phosphatidyl[N-methyl-^{3}H]choline was used in the liposome and the release of the [^{3}H]choline moiety in the reaction catalyzed by PLD is measured to indicate the amount of PLD activity present in the sample. The liposome-based PLD assay was performed as described by Brown *et al.* (286) with minor modifications. The protein content of membrane and cytosol was determined (Bio-Rad) so that 25 µg of crude membrane protein and, when required, 25 µg of cytosol could be used in each assay. Cell membranes and/or cytosol were added to tubes containing NaCl (400 mM), GTPγS (30 µM dissolved in 30 mM $MgCl_2$/20 mM $CaCl_2$), and Solution A (137 mM NaCl, 27 mM KCl, 8.1 mM Na_2PO_4, 1.5 mM KH_2PO_4, 2.5 mM EDTA at pH 7.2). This mixture was incubated in 37°C water bath with gentle shaking for 10 min. After this incubation, liposomes (as described above) were added to each tube so the final volume per tube was 100 µl. This mixture was then incubated in 37°C water bath with gentle shaking for 1 h. The reaction was terminated by the addition of 750 µl of methanol/chloroform (2:1), 250 µl of chloroform, and 350 µl of water to each tube to separate water soluble metabolites from [^{3}H]PC (287). Tubes were vortexed and centrifuged at ~ 1200 rpm for 5 min to ensure separation of aqueous from non-aqueous phases. Aliquots from the upper aqueous phase were put into scintillation vials with a scintillation cocktail of phosphate buffer (10 mM at pH 7.5), TPB (5 mg/ml) in acetonitrile, and toluene containing PPO (2,5-diphenyloxazole - 0.5 g/L) and POPOP (p-Bis[2-(-phenyloxazolyl)]-benzene - 0.2 g/L). Vials were inverted 52 times and the radioactivity was counted. Since there is phospholipase C (PLC) also present in membrane fractions, the product of PLC activity, phosphorylcholine, must be separated

from the product of PLD activity, choline, as both are soluble in aqueous media. Previous studies have used Dowex columns to separate the choline from the phosphorylcholine which is time consuming procedure. The method described efficiently extracts [^{3}H]choline from [^{3}H]phosphorylcholine as described by Fonnum (288) to ensure that the activity we count is from [^{3}H]choline as a product of PLD exclusively. Choline is soluble in the organic phase, toluene, which acts as the scintillant in this procedure. Phosphorylcholine is not soluble in this organic phase and moves into the aqueous phase. Since tritium has such a short wavelength for activating the scintillant, only the choline in the organic phase is detectable by this method. This procedure allows for the rapid and accurate quantitation of choline released from PLD activity specifically.

2.3h CELL-FREE TRITON ASSAY FOR PLD2

In order to measure PLD2 isoform activity specifically, an assay was established to measure PLD activity in the presence of Triton X-100. Triton X-100 is a detergent and a specific inhibitor of the PLD1 isoform (78,92). This assay employs the use of [^{3}H]PC in the mixed micelles and measures release of the [^{3}H]choline moiety in the reaction catalyzed by PLD to indicate the amount of PLD activity present in the sample. Protein content of membrane fractions was determined (Bio-Rad) and 50 µg of crude membrane protein was used in each assay. This PLD assay uses mixed micelles containing PIP_2 (1 µM), L-3-phosphatidyl[N-methyl- ^{3}H]choline (100 µM and about 100,000 dpm/assay), and Triton X-100 (0.1%) in place of liposomes. NaF (20 mM), which acts as a phosphatase inhibitor, was added to each tube. NaCl and GTPγS were omitted as they are not required for PLD2 activation. This mixture was then incubated in 37°C water bath with gentle shaking for 1 h. The reaction was terminated by the addition of 750 µl of methanol/chloroform (2:1), 250 µl of chloroform and 350 µl of water to each tube to separate water-soluble metabolites from [^{3}H]PC (287). Tubes were vortexed and centrifuged at ~ 1200 rpm for 5 min to ensure separation of aqueous from non-aqueous phases. Aliquots from the upper aqueous phase were put into scintillation vials with a scintillation cocktail as described above in the cell-free assay for PLD (section 2.3g). Vials were inverted 52 times and the radioactivity was counted. PLD activity present represents only PLD2 activity because of the Triton X-100 inhibition of PLD1 and the lack of GTPγS in this system.

2.4 SDS-PAGE GELS

For immunoblotting experiments, gels were run on a Bio-Rad gel apparatus using the standard procedures outlined by the company. Gels were run vertically using a Bio-Rad power supply. Membrane fractions equivalent to 20 µg of crude membrane protein were loaded and resolved on a 5% SDS-polyacrylamide stacking gel and a 10% resolving gel according to Laemmli (289). The specific composition of the gels was as follows:

5% stacking gel:

Reagent	Volume (ml)
Solution B (0.5 M Tris Base + 0.008 M EDTA)	6.3
Solution C (Acrylamide/Bisacrylamide 30%:0.8%)	4.3
10% SDS	0.25
66% sucrose	2.5
Cold distilled water	9.15
Ammonium persulfate (0.3 g/20 ml)	2.5
N,N,N',N'-Tetramethylethylenediamine	0.025

10% resolving gel:

Reagent	Volume (ml)
Solution A (1.5 M Tris Base + 0.008 M EDTA)	12.5
Solution C (Acrylamide/Bisacrylamide 30%:0.8%)	16.5
10% SDS	0.5
66% sucrose	5.0
Cold distilled water	10.45
Ammonium persulfate (0.3 g/20 ml)	5.0
N,N,N',N'-Tetramethylethylenediamine	0.05

2.5 WESTERN BLOT ANALYSIS

Proteins were electrotransferred from SDS-PAGE gels to PVDF membranes for 4 h at 300 mA. Immunoblots were blocked overnight with a 5% powdered milk buffer solution. The membranes were washed with PBS-Tween 20 and then incubated with either RhoA antibody (1:1500), ARF antibody (1:5000), PKCα antibody (1:2000), or PKCζ antibody (1:280) in blocking buffer. Membranes were then washed with PBS-Tween 20. Membranes were incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit antibody, washed again, and then soaked in a chemiluminescence reagent (ECL-Plus kit from Amersham) for 5 min and then exposed to film. Membrane exposure to film ranged from 1 sec to 2 min depending on the antibody used and the signal obtained. For all blots, at least two exposures were done to ensure the accurate interpretation of results. Western blot images were captured using Storm 840 scanner and analyzed with the Macintosh application of ImageQuant v.1.2.

3. RESULTS

Thyroid hormones are important regulators of metabolism in the body. Thus, an understanding of the mechanism by which thyroid hormone production is regulated is crucial to subsequently establishing methods to treat thyroid disorders. The goal of this thesis was to determine those factors which modulate thyroid function with a focus on the role of phospholipase D as this enzyme is activated by both major TSH-mediated signalling pathways. Since PLD is the focus of my research, I wanted to identify those agonists capable of activating PLD and determine via which pathway, PKA and/or PKC, the activation occurred. Through this approach, the physiological mediators of these pathways could be better understood and manipulated. We initially tested lysophosphatidic acid for its PLD activating capacity because its role as an activator of PLD has been established in other cell systems.

3.1 THE EFFECTS OF LYSOPHOSPHATIDIC ACID ON PLD ACTIVITY IN FRTL-5 THYROID CELLS

Previous studies have shown that lysophosphatidic acid (LPA) can activate PLD in vitro in HL60 cells (61) and fibroblasts (57) which establishes a role for LPA in cell signalling. The transphosphatidylation reaction, where PLD converts PC to phosphatidylethanol (PEt) in the presence of ethanol, has been used by ourselves and other groups to establish PLD activity in different cell systems (56). The transphosphatidylation reaction, with slight modifications, has been used in FRTL-5 thyroid cells to demonstrate PLD activity.

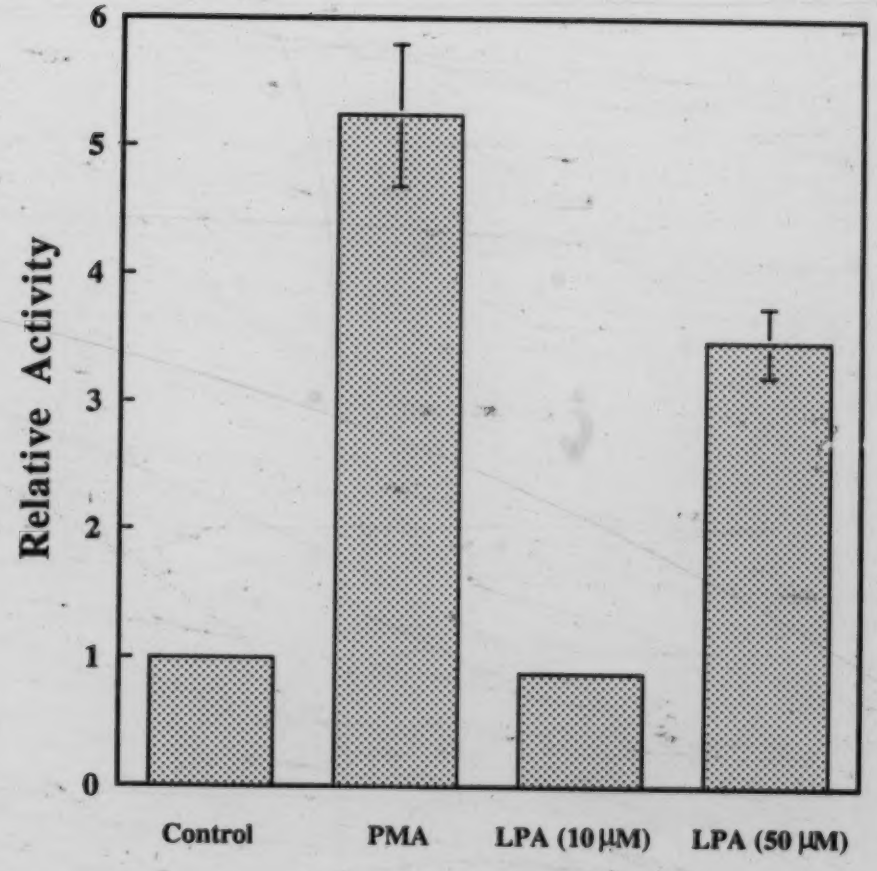
To establish this assay in our laboratory, the ability of known PLD agonists to activate PLD were tested in FRTL-5 thyroid cells. Cells were treated as described in Materials and Methods, incubated in the presence of ethanol and the lipids were extracted and run on thin layer chromatography (TLC) plates for separation. Phosphatidylethanol and phosphatidylcholine bands were assessed to determine the PLD activity present. The results are summarized in Fig. 3. Significantly increased PLD activity (approximately 3.5-fold above control values $P<0.05$, ANOVA) was seen with high concentrations of LPA (50 μM), while lower concentrations (10 μM) of LPA were unable to activate PLD. Phorbol-myristate-acetate (PMA- 100 nM) also served as a positive control in this experiment as it is a known activator of PLD via PKC activation.

This study was the first to show that PLD could be activated by LPA in thyroid cells. It is not clear from the results through which pathway LPA is able to activate PLD. As described above, there are two pathways in FRTL-5 thyroid cells which lead to the activation of PLD, one through cAMP and PKA, the other through PKC. In other cell systems, LPA has been shown to directly inhibit the activity of adenylyl cyclase and cAMP accumulation (260,265). Our laboratory has demonstrated that both

FIGURE 3

The effects of LPA on PLD activity

FRTL-5 thyroid cells were treated with [^{3}H]myristate for 2 h. Excess label was washed away and cells were treated with LPA (10 or 50 μM), PMA (100 nM), or as controls for 30 min. Cells were scraped from tissue culture dishes with methanol and lipids were extracted. Lipids were run on TLC plates for separation and the PC and PEt bands were cut from plastic-backed silica gel plates. The activity from these bands was counted. PEt was calculated as a percentage of PC and these values were converted to relative activity as compared to control. Results are the mean ± SD (where large enough to be shown) from three independent experiments.


Relative Activity
6
5
4
3
2
1
0
Control
PMA
LPA (10 μM)
LPA (50 μM)

10 and 50 μM LPA inhibits TSH-mediated cAMP production in FRTL-5 thyroid cells (290). Since only 50 μM LPA activates PLD in these cells, we can conclude that LPA is acting through a direct inhibition of adenylyl cyclase. In addition, it is not clear how LPA is activating PLD since LPA inhibits cAMP production. LPA could act through the PKC pathway to activate PLD or, LPA could cause tyrosine phosphorylation of PLD and activate it, as LPA has been shown to induce phosphorylation of tyrosine residues of PLD in other cells (61). Further elucidation of the involvement of LPA in these pathways will clarify these results and help us understand the signalling role that LPA plays in these cells. Since PLD is activated by LPA, it will also be of interest in defining these signalling cascades.

3.2 THE CHARACTERIZATION OF PHOSPHOLIPASE D IN FRTL-5 THYROID CELLS IN THE CELL-FREE ASSAY

Although PLD is widely distributed in mammalian cells (56), its biological role has not been clearly defined. As a prelude to establishing the role of PLD in FRTL-5 thyroid cells, the identification of PLD isoforms and their activators was studied. The two mammalian PLD isoforms identified to date, PLD1 and PLD2, potentially play different roles in the cell because of their different requirements for activation (78,79). Since only PLD1 requires small G-proteins and GTPγS for activation (56,69), we can use this property to distinguish the activities of each isoform. The involvement of G-proteins and the subcellular location of PLD isoforms implicate each isoform in different cellular events. In FRTL-5 thyroid cells, TSH has been shown to activate PLD through both a PKA- and PKC-mediated pathway (53,55). Since TSH-mediated PLD1 activation is dependent upon small G-proteins, the context of cellular events associated with these small G-proteins, both upstream and downstream of PLD1 activation can be defined. For example, RhoA, which is involved in cytoskeletal reorganization, is translocated to the membrane fraction upon TSH stimulation. Thyroid hormone production, which is also activated by TSH, thus, may involve cytoskeletal movement. Therefore, it is important to know which PLD isoform(s) are present in FRTL-5 cells and their regulation to establish other cellular events that may be involved with these processes.

The cell-free assay used to determine PLD activity in FRTL-5 cells has been used by others (286) and was adapted to these cells with slight modifications. This assay measures the amount of [^{3}H]choline released from PC as it is converted by PLD to PA. This indicates the amount of PLD activity present. Cells were treated, harvested and membrane and cytosolic fractions separated as described in Materials and Methods. The cytosol, membranes, and a combination of the two were assayed for

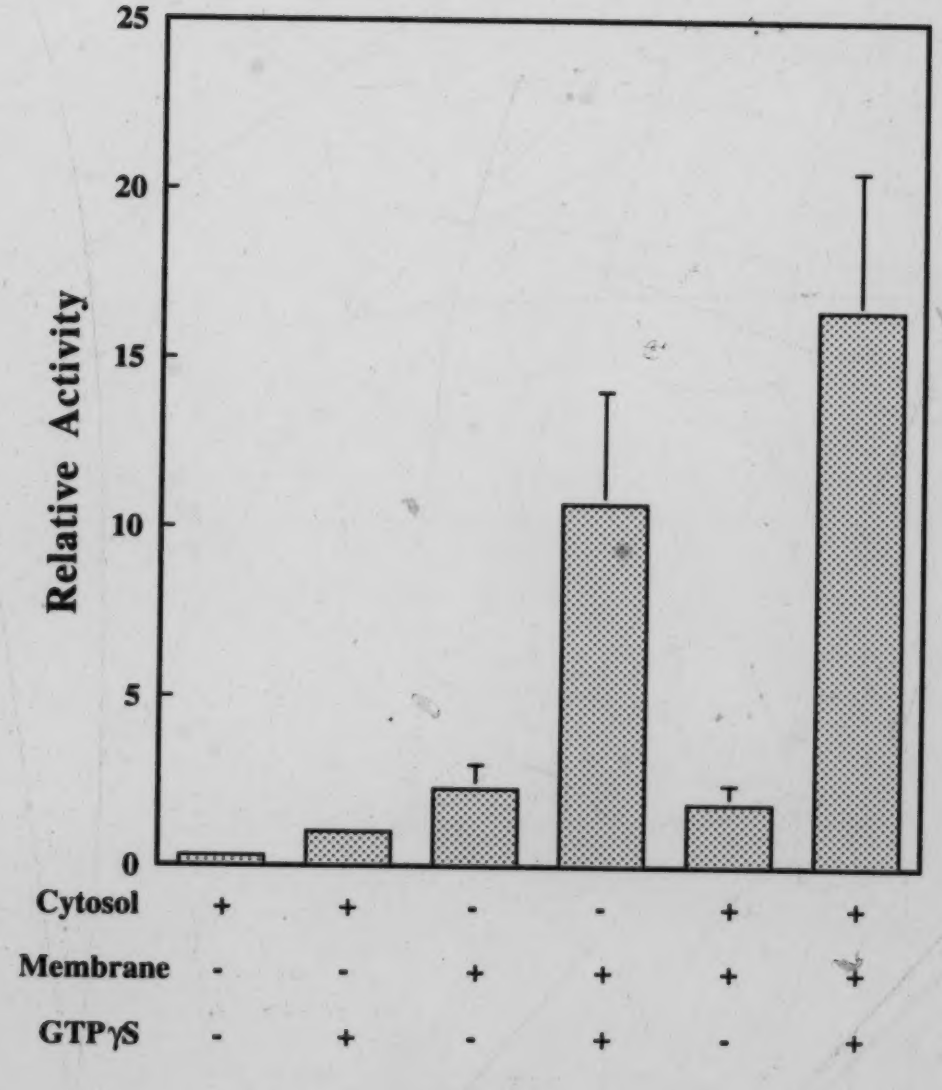
PLD activity with the addition of a liposomes containing [^{3}H-methyl]PC, PIP_2, and PE. The reaction was performed as described in Materials and Methods.

PLD activity in the membrane and cytosolic fractions of FRTL-5 thyroid cells in the presence and absence of GTPγS is shown in Fig. 4. The cytosolic fraction revealed minimal PLD activity in the presence or absence of GTPγS. The membrane fraction was associated with very little activity in the absence of GTPγS, but in its presence, PLD activity increased by approximately 5-fold ($P<0.05$). This increase is likely due to the presence of small molecular weight G-proteins associated with the membrane prior to stimulation or to imperfect separation of membrane and cytosolic fractions. Since this PLD activity is stimulated by GTPγS, this is consistent with PLD1 activity. When membrane and cytosolic fractions were combined, no increase in PLD activity compared to either fraction alone was observed in the absence of GTPγS. However, in the presence of GTPγS, the combined membrane plus cytosolic fraction was associated with a maximal 8-fold increase in PLD activity ($P<0.05$, ANOVA). This confirms that components from both the cytosolic and membrane fractions are essential for the maximal activation of PLD. This is consistent with the PLD requirement for small molecular weight G-proteins found in the cytosol (73-75) and thus, PLD1. The results also indicate that maximal PLD1 activity is additionally dependent on GTPγS. When taken together, the requirement for small G-proteins and GTPγS are consistent with the concept that PLD1 is the predominant PLD isoform in FRTL-5 thyroid cells.

FIGURE 4

The effects of GTPγS on PLD activity in the cytosolic (25 μg protein), membrane (25 μg protein), and combined fractions

FRTL-5 thyroid cells were harvested and cytosolic and membrane fractions were isolated. PLD activity was determined using liposomes of PC, PE, and PIP_2 (12 μM). The activities are normalized to the specific activity in cytosol stimulated with 30 μM GTPγS and this value represents approximately 6% of the [^{3}H]PC converted to [^{3}H]choline. Results are the mean ± SD (where large enough to be shown) from four independent experiments.


25
20
15
10
5
0
Relative Activity
Cytosol + + - - + +
Membrane - - + + + +
GTPγS - + - + - +

3.3 THE EFFECT OF ADENOSINE DEAMINASE ON PLD ACTIVITY

Adenosine is present in many cell types (291,292) and can play a role in signal transduction. About ten years ago, studies were done that implicated a variety of receptor-mediated actions to the purine nucleoside adenosine (293). The adenosine receptor can interact with guanine nucleotide binding proteins (294,295), like ARF and RhoA, which are known to play a role in PLD1 activation in many cell types (56,69). In FRTL-5 thyroid cells, PLD1 may be activated by these small G-proteins after TSH stimulation. However, the presence of adenosine may interfere with the ability of these small G-proteins to activate PLD1. In addition to the adenosine receptor binding of small G-proteins, adenosine is also able to inhibit adenylyl cyclase in some tissues, particularly adipocytes (296). Since TSH stimulates the activation of adenylyl cyclase and the accumulation of cAMP leading to PLD activation, inhibition or downregulation of adenylyl cyclase by adenosine could block this activation and produce inaccurate results. If adenosine blocks cAMP generation, a PLD response may not be seen or it may appear smaller than if adenosine did not interfere. Thus, we attempted to eliminate adenosine from the cell system to determine if adenosine was downregulating TSH-mediated PLD1 activation.

In order to determine the physiological effects of adenosine binding to its receptor, FRTL-5 cells had to first be depleted of their endogenous adenosine. This is usually accomplished by the addition of adenosine deaminase (adenosine aminohydrolase) which converts adenosine to inosine. Inosine is a very weak activator of adenosine receptors (293). Although adenosine deaminase is not 100% effective in eliminating endogenous adenosine, it does provide a means to determine how adenosine affects certain cell signalling systems. Thus, adenosine deaminase was added to FRTL-5 cells prior to the addition of TSH to inactivate the adenosine that may be present. PLD1 activity was measured in the presence and absence of adenosine

deaminase to determine if this would allow for TSH-mediated PLD1 activation to reach a maximal level. However, addition of adenosine deaminase did not have a consistent effect on TSH-mediated PLD1 activation in FRTL-5 thyroid cells (data not shown) and there is no evidence that adenosine enhanced the PLD1 response to TSH. Thus, we determined that adenosine and its receptor do not play a significant role in TSH-mediated PLD1 activation.

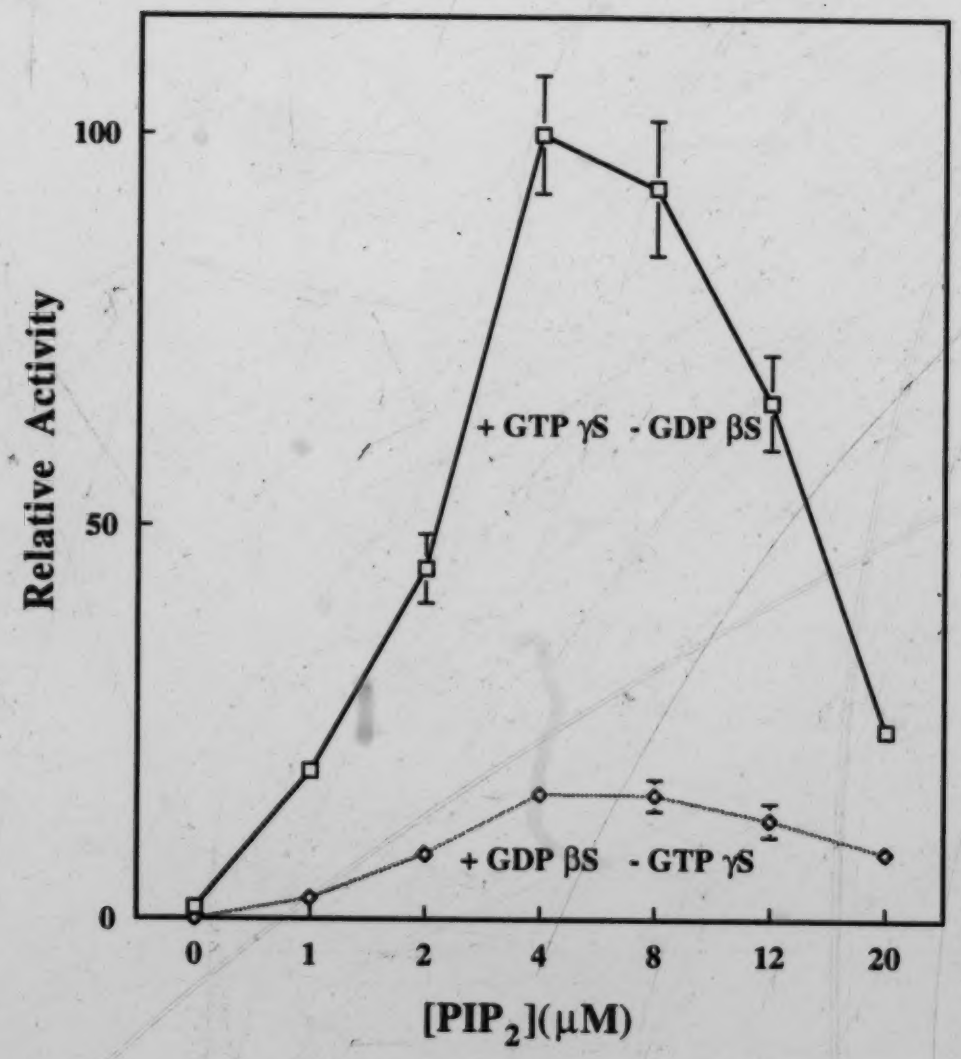
3.4 THE REQUIREMENT OF PHOSPHOLIPASE D1 AND 2 FOR PIP_2 IN FRTL-5 THYROID CELLS

We have shown that PLD1 is the predominant isoform in FRTL-5 thyroid cells. To further confirm this concept and to determine the relative concentrations of the two isoforms, the cell-free PLD assay was performed with varying PIP_2 concentrations. Previous studies have shown that PLD is dependent on PIP_2 for activation (56,69). Both PLD1 and PLD2 have an absolute requirement for PIP_2 for activation. In order to establish the relative concentrations of the two PLD isoforms present, the cell-free assay system was used to generate a PIP_2 concentration curve for PLD activity. This experiment also further characterizes the predominance of the PLD1 isoform in FRTL-5 thyroid cells by demonstrating its dependence on small G-proteins for activation. The cell-free assay was performed, using membrane fractions only, with varying concentrations of PIP_2 in the presence of GTPγS or GDPβS, which is an inhibitor of small molecular weight G-proteins (56,69). GDPβS is an non-hydrolyzable, non-phosphorylatable form of GDP that is bound by small molecular weight G-proteins rendering them inactive. The assay was done in the presence of GDPβS to establish that PLD activity in the absence of GTPγS is not due to the presence of small amounts of activated G-proteins which can activate PLD1, but it is, in fact, PLD2 activity. As shown in Fig. 5, the PLD activity in the presence of GTPγS represents total PLD activity in the membranes since the components necessary for both PLD1 and PLD2 activation are present. PLD activity in the presence of GDPβS represents only PLD2 activity since it is independent of both small G-proteins and GTPγS (79) and these factors are necessary for PLD1 activity. Since only PLD1 is dependent on G-proteins for activation, we ensure that only PLD1 activity is eliminated. There has been some suggestion that PLD2 may be activated by ARF under certain conditions (81), but the results from these experiments are inconclusive and utilize cells transfected with PLD2

FIGURE 5

The effects of GTPγS and PIP_2 on PLD activity

Membrane fractions of FRTL-5 thyroid cells were used in the cell-free PLD assay in the presence of GTPγS or GDPβS using different concentrations of PIP_2 as indicated. The solid line indicates total PLD activity in the presence of GTPγS, while the dotted line represents PLD activity (PLD-2, see text) in the presence of GDPβS. PLD activity using liposomes containing 4 μM PIP_2 plus GTPγS is expressed as 100%. This value represents approximately 15% of the [^{3}H]PC converted to [^{3}H]choline. Results are the mean ± SD (where large enough to be shown) from three independent experiments.


Relative Activity
100
50
0
+ GTP γS - GDP βS
+ GDP βS - GTP γS
0
1
2
4
8
12
20
[PIP2](μM)

which may not represent the response of endogenous PLD2. In addition, several studies have been done to indicate that PLD1, but not PLD2, is responsive to small G-proteins and GTPγS (56,69). Some researchers suggest that both isoforms of PLD can be activated *in vivo* by certain agonists (156), but the degree to which PLD1 is activated is almost 20-fold greater than for PLD2. Despite this controversial data, the vast majority of results suggest that only PLD1 requires small G-proteins and GTPγS for activation, particularly *in vitro*. With the addition of GDPβS without GTPγS, the PLD activity observed represents only that of PLD2 as we have inhibited the activators of PLD1. This PLD2 activity is consistent with about 20% of the total PLD activity in the membrane fraction. These results further support our previous finding that PLD1 is the predominant isoform and comprises about 80% of the total PLD present in FRTL-5 thyroid cells as estimated by the areas under the curve. Although PLD1 appears to be the predominant PLD isoform present in FRTL-5 thyroid cells, the role of PLD2 in TSH-mediated PLD activation deserves to be assessed.

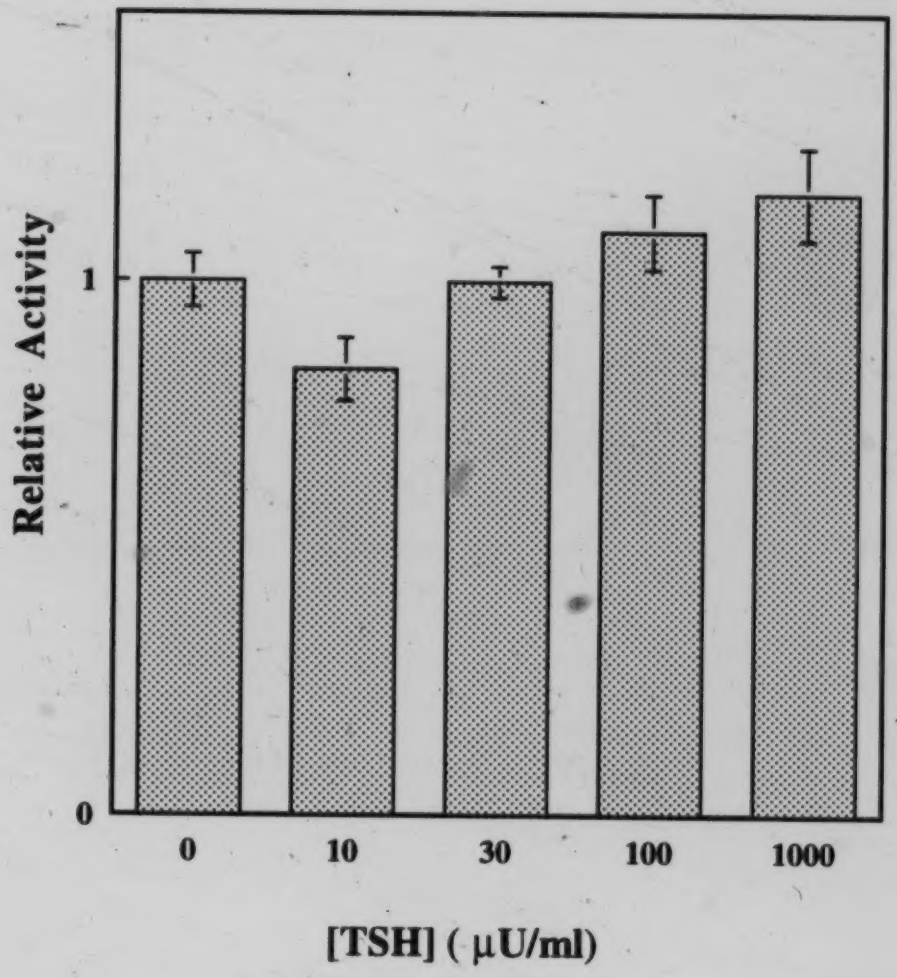
3.5 THE EFFECT OF TSH ON PLD2 ACTIVITY IN FRTL-5 THYROID CELLS

In some cell systems it has been proposed that the PLD isoforms, PLD1 and PLD2, have different biological roles due to their differential requirements for activation and their different subcellular locations (78,79,86,87,92). Therefore, our next step was to determine the role of PLD2 in FRTL-5 thyroid cells and the possibility of its involvement in the pathways mediated by TSH. We assayed PLD2 activity specifically using a cell-free assay, which we have named the Triton assay. This assay measures PLD2 activity using mixed micelles containing [^{3}H-methyl]PC, PIP_2, and Triton X-100. Triton X-100 is a specific inhibitor of PLD1 (78,92) and, when used in this assay, allows for the measurement of PLD2 activity only. This assay was performed as described in Materials and Methods in the absence of GTPγS as it is not required in our assay for PLD2 activity due to its independence of small G-proteins. PLD activity in membrane fractions was measured in the presence of TSH at concentrations up to 1 mU/ml. TSH was unable to activate PLD2 activity at any concentration as shown in Fig. 6. These findings suggest that TSH-mediated PLD activation does not involve PLD2. In several cell lines, PLD2 has been shown to be constitutively active and unresponsive to external agonists (79) which is consistent with our findings in FRTL-5 thyroid cells.

FIGURE 6

The effect of TSH on PLD2 activity

FRTL-5 thyroid cells were incubated with different concentration of TSH for 20 min as indicated. Membrane and cytosolic fractions were separated and the membrane fractions were used in the cell-free Triton assay using micelles containing PC, PIP_2 and Triton X-100 to measure PLD2 activity. Results are expressed relative to the untreated control and this value represents approximately 3% of the [^{3}H]PC converted to [^{3}H]choline. Results are the mean ± SD from three independent experiments.


Relative Activity
1
0
0
10
30
100
1000
[TSH] (μU/ml)

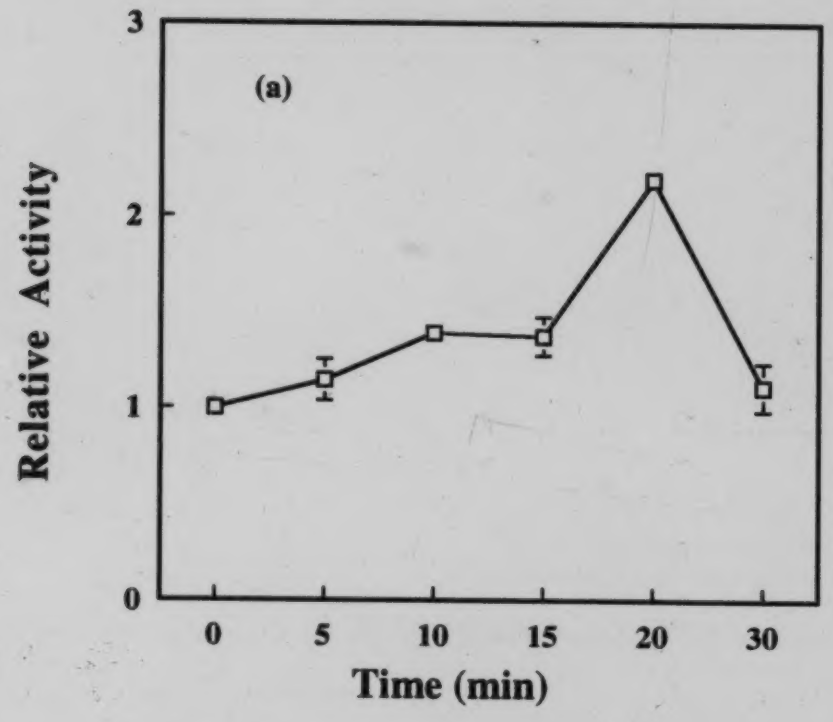
3.6 THE EFFECT OF TSH ON ACTIVATION OF PLD1 IN FRTL-5 THYROID CELLS

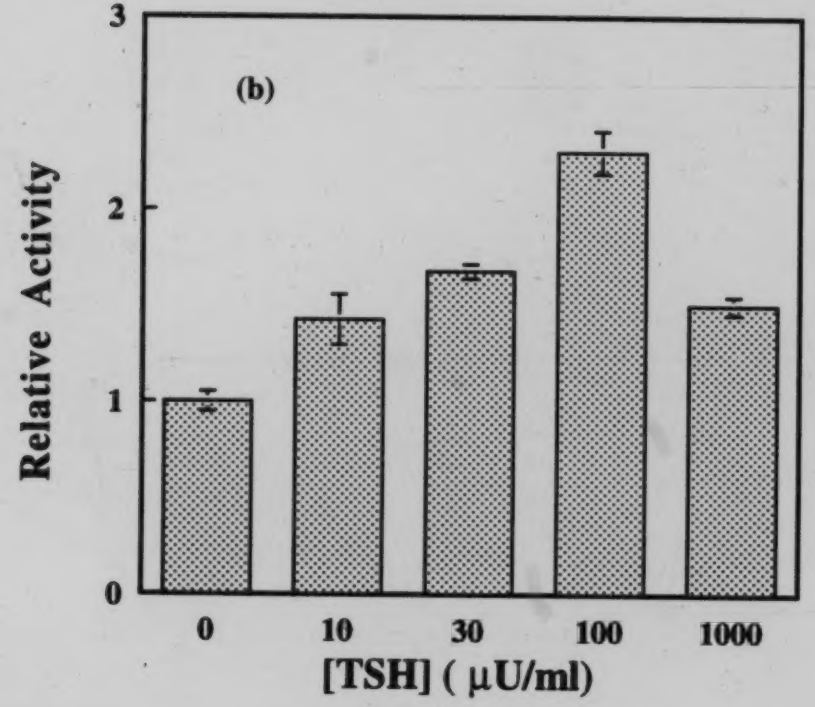
Since TSH-mediated PLD activation does not involve PLD2 activation, we determined whether PLD1 is activated by TSH in FRTL-5 thyroid cells. To determine whether TSH activates PLD1, cells were incubated with TSH (100 μU/ml) at different time points (5, 10, 20 and 30 min). PLD activity was measured in the membrane fractions in the presence of GTPγS as described in Materials and Methods. Fig. 7a shows that TSH incubation for 20 min produces the maximum stimulation of PLD1 activity greater than 2-fold above control values ($P<0.01$, ANOVA). Time points greater than 20 min showed almost a return to basal levels. Since this PLD1 activation is GTPγS-dependent, we suggest that PLD activation in these experiments is exclusively PLD1. Using the 20 min incubation time, a TSH dose-response curve for PLD1 activation was generated (Fig. 7b). TSH concentrations above 10 μU/ml were all able to significantly increase PLD1 activity by a 1.7 to 2.3-fold increase ($P<0.01$, ANOVA). In other cell systems, PLD1 has been shown to be responsive to external agonist stimulation (56,69) which is similar to our response in FRTL-5 thyroid cells. This is the first study to characterize the specific isoforms of PLD present in FRTL-5 thyroid cells and demonstrate their differential regulation by TSH. In light of this evidence, the role of small G-proteins was examined to determine their role in TSH-mediated PLD activation.

FIGURE 7

Activation of PLD1 by TSH

FRTL-5 thyroid cells were incubated with TSH (100 μU/ml) for the times indicated (a), or for 20 min with different concentrations of TSH (b). Membrane fractions were used in the cell-free PLD assay using liposomes in the presence of GTPγS. Results are expressed relative to the untreated control and this value represents approximately 10% of the [^{3}H]PC converted to [^{3}H]choline. Results are the mean ± SD (where large enough to be shown) from three independent experiments except for 30 min point in (a) where means ± ranges of two independent experiments are shown.


(a)
Relative Activity
3
2
1
0
0
5
10
15
20
30
Time (min)


(b)
Relative Activity
3
2
1
0
0
10
30
100
1000
[TSH] (μU/ml)

3.7 THE EFFECT OF TSH ON RHOA AND ARF TRANSLOCATION TO THE MEMBRANE FRACTION IN FRTL-5 THYROID CELLS

PLD1 dependence on small molecular weight G-proteins, including ARF and RhoA, has been characterized in several cell types (56,69). Both of these small G-proteins have been shown to activate PLD1, typically in a synergistic manner (91,92). These small G-proteins are usually found in the cytosol prior to stimulation by agonist after which they are translocated to the membrane fraction (56,69). To establish that TSH stimulation of PLD1 is dependent on the small molecular weight G-proteins ARF and RhoA, a time course (0-20 min) of the effect of TSH (100 μU/ml) on RhoA and ARF translocation to the membrane fraction was determined. Fig. 8a shows that the maximal translocation of RhoA to the membrane fraction occurred after a 10 min incubation with TSH. A 2.5-fold increase in membrane-associated RhoA was observed compared to controls ($P<0.01$, ANOVA). Fig. 8b shows the maximal translocation of ARF to the membrane fraction also occurred after a 10 min incubation with TSH and produced a 2.3-fold increase in membrane-associated ARF as compared to controls ($P<0.01$, ANOVA). Using the 10 min incubation time, dose-response curves using TSH (10-1000 μU/ml) for RhoA and ARF translocation are shown in Fig. 9a and b. TSH at 100 μU/ml increased the membrane content of RhoA by 1.9-fold above control values ($P<0.03$, ANOVA). Similarly, TSH at 100 μU/ml increased the membrane content of ARF by 1.7-fold above control values ($P<0.04$, ANOVA). Corresponding Western blots are shown in Fig. 10. These results indicate the involvement of RhoA and ARF in TSH-mediated PLD1 activation. RhoA and ARF translocation appears to occur prior to PLD1 activation suggesting that the translocation of RhoA and ARF mediates the activation of PLD1. These results have helped to establish a model of TSH action in FRTL-5 thyroid cells. Further elucidation of the pathways involved (PKA and/or PKC) is the next critical step for understanding the

FIGURE 8

Time-dependent effect of TSH on RhoA and ARF translocation to the membrane fraction

FRTL-5 thyroid cells were incubated with 100 μU/ml TSH for the times indicated. Membrane fractions were analyzed for (a) RhoA and (b) ARF by Western blotting. Images were captured using Storm 840 scanner and analyzed with the Macintosh application of IamgeQuant v.1.2. Results are expressed relative to the untreated control. Results are the mean ± SD from three independent experiments.

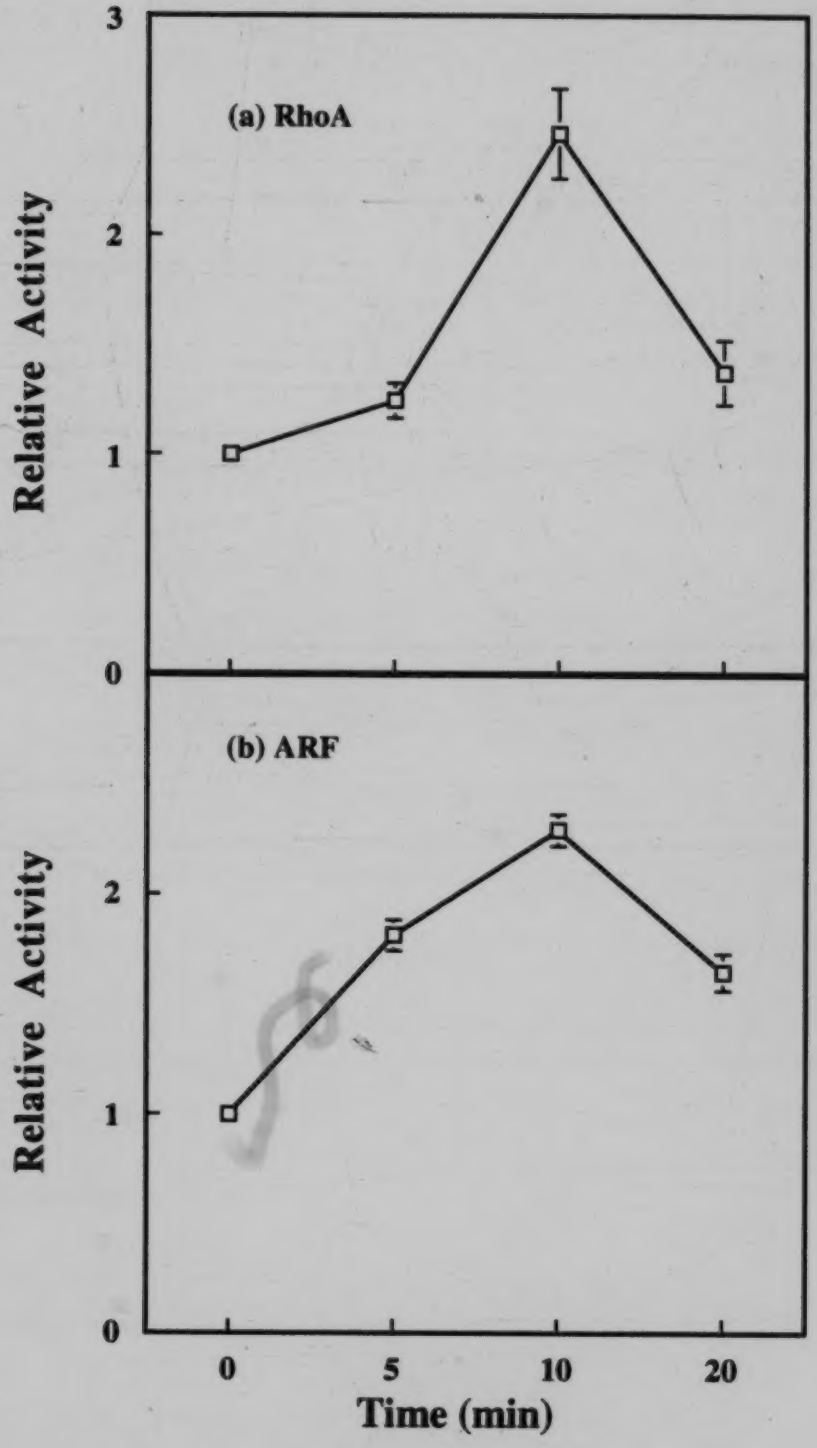

(a) RhoA
Relative Activity
3
2
1
0
(b) ARF
Relative Activity
2
1
0
0
5
10
20
Time (min)

FIGURE 9

The effects of TSH concentration on the translocation of RhoA and ARF to the membrane fraction

FRTL-5 thyroid cells were incubated with different concentrations of TSH for 10 min as indicated. Membrane fractions were analyzed for (a) RhoA and (b) ARF by Western blotting. Images were captured using Storm 840 scanner and analyzed with the Macintosh application of IamgeQuant v.1.2. Results are expressed relative to the untreated control. Results are the mean ± SD from three independent experiments.

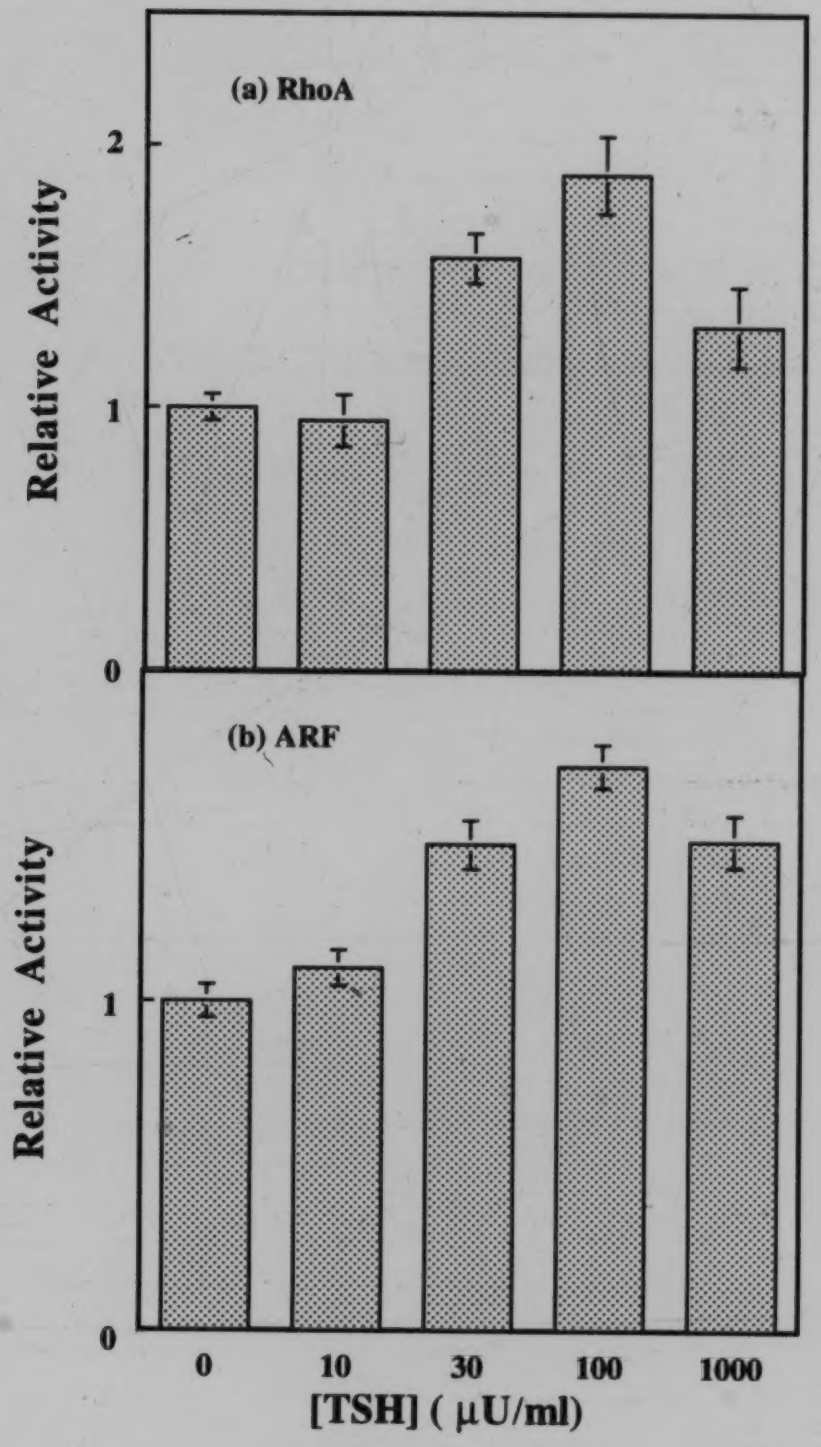
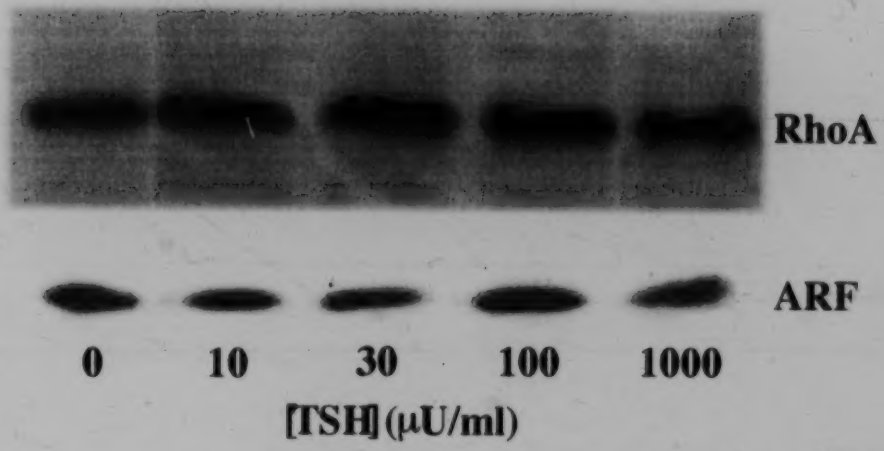

(a) RhoA
Relative Activity
2
1
0
(b) ARF
Relative Activity
1
0
0
10
30
100
1000
[TSH] (μU/ml)

FIGURE 10

The effects of TSH concentration on RhoA and ARF translocation to the membrane fraction

Representative Western blots of RhoA and ARF translocation to the membrane fraction after treatment with different concentrations of TSH as indicated.


RhoA
ARF
0
10
30
100
1000
[TSH] (μU/ml)

nature of TSH action in these cells.

3.8 THE EFFECT OF PROTEIN KINASE A AND PROTEIN KINASE C INHIBITORS ON TSH-MEDIATED PLD1 ACTIVITY

TSH activates two separate pathways in FRTL-5 thyroid cells, one involving cAMP and PKA (55), and the other involving diacylglycerol and PKC (53). To determine whether the PKA and/or the PKC pathways are involved in TSH-mediated PLD1 activity, we utilized specific PKA and PKC inhibitors separate or together to block either one or both of the pathways. Dideoxyadenosine (DDA - 5 nM) was chosen as an inhibitor of the PKA pathway (55). FRTL-5 cells were incubated with DDA for 10 min prior to the addition of TSH (100 μU/ml) for 20 min as described in Materials and Methods. PLD activity was measured in membrane fractions using the cell-free assay. As shown in Fig. 11, cells incubated with DDA prior to TSH blocked TSH stimulation of PLD1 ($P< 0.01$, ANOVA) compared to controls.

Chelerythrine (1 μM) was used as an inhibitor of the PKC pathway (53). Cells were also incubated with chelerythrine for 10 min prior to the addition of TSH (100 μU/ml) for 20 min and a similar inhibition in TSH-mediated PLD1 activity was observed ($P<0.01$, ANOVA) (Fig. 11). Cells incubated with both inhibitors prior to the addition of TSH (100 μU/ml) did not produce further inhibition of PLD1 activity compared with each individual inhibitor alone. These results indicate that activation of either the PKA or PKC pathway leads to TSH-mediated PLD1 activation, but that both pathways are required for maximum PLD1 activation. Preliminary results depicting the translocation of RhoA and ARF to the membrane fraction after incubation with TSH in the presence of these inhibitors is shown in Fig. 12. These results indicate that TSH-mediated translocation of both RhoA and ARF to the membrane is inhibited by both DDA and chelerythrine. Although the inhibition of RhoA translocation by both inhibitors is convincing, the inhibition of ARF translocation after treatment with DDA is not as clear. Further studies will be needed to verify the ARF response to DDA.

FIGURE 11

The effects of PKA and PKC inhibitors on TSH-mediated PLD1 activation

FRTL-5 thyroid cells were incubated with PKA (DDA - 5nM) and/or PKC (chelerythrine - 1 μM) inhibitors for 10 min prior to the addition of TSH (100 μU/ml) for 20 min. Membrane fractions were isolated and PLD activity was measured using the cell-free PLD assay. Results are expressed relative to the untreated control. Results are the mean ± SD from three independent experiments.

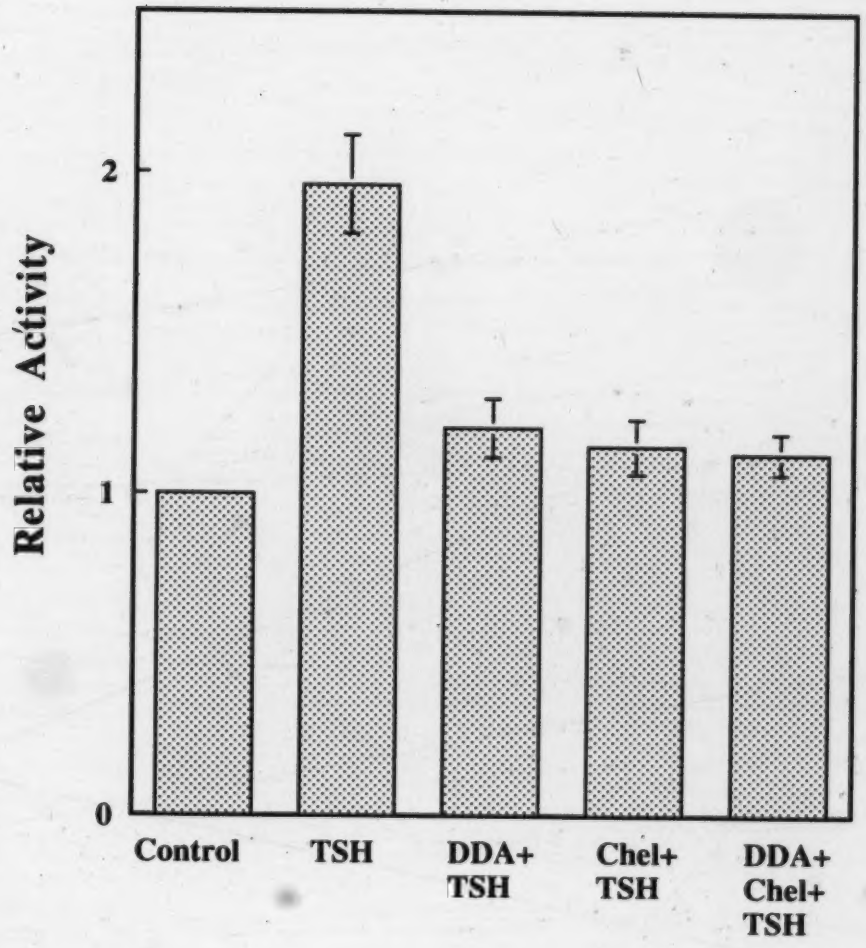

Relative Activity
2
1
0
Control
TSH
DDA+ TSH
Chel+ TSH
DDA+ Chel+ TSH

FIGURE 12

The effects of PKA and PKC inhibitors on RhoA and ARF translocation to the membrane fraction

FRTL-5 thyroid cells were incubated with DDA (5 nM) and/or chelerythrine (1 μM) for 10 min prior to the addition of TSH (100 μU/ml) for 10 min. Membrane fractions were analyzed for RhoA and ARF by Western blotting. A representative Western blot for RhoA and ARF translocation to the membrane fraction is shown.

RhoA
ARF
Control
TSH
DDA +TSH
Chel +TSH
DDA +Chel +TSH

Although these results must be confirmed, they do support our findings that TSH-mediated PLD1 activation occurs through both the PKA and PKC pathways. In addition to the use of inhibitors to determine the involvement of the PKA and PKC pathways in TSH-mediated PLD1 activation, the activation of PLD1 by agonists of each of the individual pathways (ie. PKA and PKC) was determined.

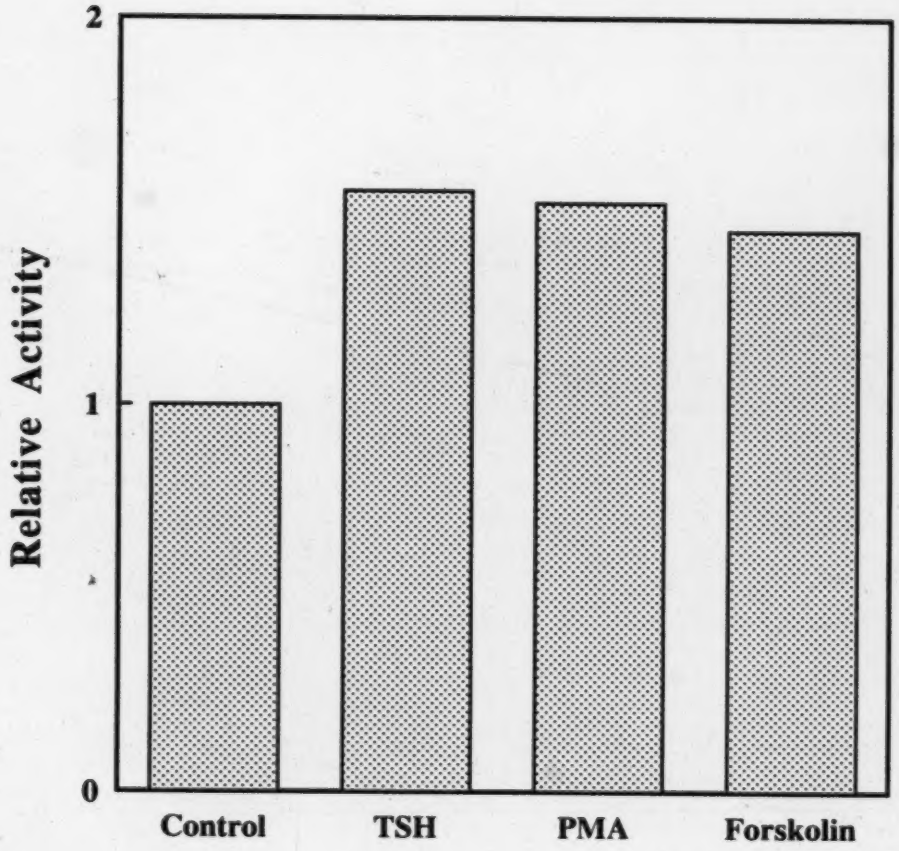
3.9 THE EFFECT OF PMA AND FORSKOLIN ON PLD1 ACTIVITY

Since both the PKA and PKC pathways appear to be involved in TSH-mediated PLD1 activity, we wanted to determine if specific PKA and PKC agonists could stimulate PLD1 activation using our cell-free PLD assay. Our studies have shown that TSH is able to activate PLD1. Forskolin, which activates adenylyl cyclase and leads to the accumulation of cAMP, was used as a PKA agonist. Phorbol-myristate-acetate (PMA), which activates PKC, was also used. Preliminary results show that either forskolin (1 μM) and PMA (100 nM) were able to stimulate PLD activity to a similar level of activation exhibited by TSH (Fig. 13). This could indicate that both pathways are involved in the activation of PLD1 in FRTL-5 thyroid cells. In addition to the effects of PKA and PKC activators on PLD1, similar activators were used to determine RhoA translocation to the membrane fraction.

FIGURE 13

The effects of PKA and PKC agonists on PLD activation

FRTL-5 thyroid cells were incubated with TSH (100 μU/ml), forskolin (1 μM) or PMA (100 nM) for 20 min. Membrane fractions were isolated and used in the cell-free PLD assay in the presence of GTPγS. Results are expressed relative to the untreated control.


2
1
0
Relative Activity
Control
TSH
PMA
Forskolin

3.10 RHOA TRANSLOCATION IN RESPONSE TO PMA AND DIBUTYRYL cAMP

The small molecular weight G-proteins RhoA and ARF are necessary for the activation of the PLD1 isoform. Both proteins are translocated to the membrane fraction upon stimulation by TSH and lead to the activation of PLD1. However, it is not clear which of the two pathways stimulated by TSH, PKA and/or PKC, are involved in the translocation of RhoA and ARF to the membrane fraction. To determine which pathway(s) are involved, we stimulated FRTL-5 thyroid cells with specific PKA or PKC activators. Dibutyryl cAMP (dbcAMP) is an analog of cAMP and, thus, an activator of the PKA pathway while PMA activates the PKC pathway as discussed previously. Preliminary results show that RhoA translocates to the membrane after stimulation with PMA (Fig. 14). Results with dbcAMP showed a modest increase in RhoA translocation. Since both PMA and dbcAMP appear to cause RhoA translocation, it is possible that both pathways are involved in activation of PLD1 through RhoA. Further studies are necessary to determine the relative contributions of each pathway to the translocation of RhoA in FRTL-5 thyroid cells.

FIGURE 14

The effects of PMA and dbcAMP on RhoA translocation to the membrane fraction

FRTL-5 thyroid cells were treated with PMA (100 nM), dbcAMP (100 nM), or TSH (100 μU/ml) for 10 or 20 min as indicated. Membranes fractions were analyzed for RhoA by Western blotting. A representative Western blot for RhoA translocation to the membrane fraction is shown.

Control 10 20 10 20 10 20

PMA TSH dbcAMP

3.11 PKC ISOFORM TRANSLOCATION IN RESPONSE TO TSH IN FRTL-5 THYROID CELLS

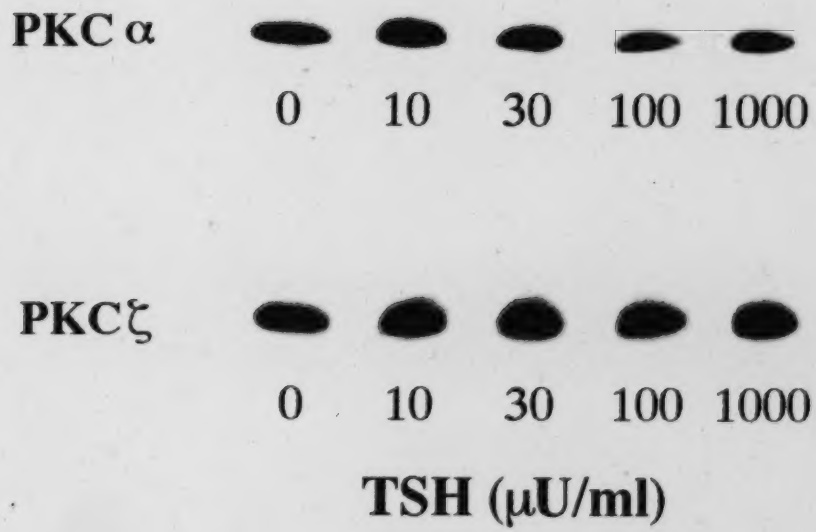
PKC has been shown to play a major role in the activation of PLD in many cells (72). The α- and β-PKC isoforms have been shown to directly activate PLD1 (91,92). While other isoforms have been unable to do so, a solitary report implicates PKCδ in the activation of PLD (241). The role of PKC isoforms in TSH-mediated PLD1 activation has been discussed previously in section 1.14. In FRTL-5 thyroid cells, the presence of PKC isoforms was determined and include the α-, δ-, ε-, and ζ-PKC isoforms (256). Our group demonstrated that TSH (100 μU/ml) and PMA (100 nM) caused the translocation of the α-, ε-, and ζ-PKC isoforms to the membrane fraction (50). PKC-δ was activated after chronic TSH exposure. The observations from this study indicate that the PKC isoforms present in FRTL-5 thyroid cells are regulated by TSH and phorbol ester *in vitro*. Activation of the PKC pathway leads to inhibition of steps involved in thyroid hormone synthesis *in vitro* (3,297), but the mechanism by which this occurs has not been defined. Thus, the role of PKC in mediating the actions of PLD1 could be very important to understanding how PLD regulates thyroid function.

My own studies have confirmed the presence of the α– and ζ–PKC isoforms in basal and TSH stimulated FRTL-5 thyroid cells (Fig. 15). Although attempts were made to quantify increases or decreases in these isoforms in the membrane fraction after TSH stimulation, the results did not show a significant increase of either PKCα or ζ. Previous studies in our lab have shown that TSH stimulates the translocation of the PKCα, ε and ζ isoforms after 30 min of TSH (100 μU/ml) stimulation (50). For the present study, FRTL-5 cells were incubated with TSH (100 μU/ml) for 10 min because maximal ARF and RhoA translocation were observed at this time point. No significant PKC isoform translocation was observed at this time in the previous studies (50).

FIGURE 15

The effects of TSH on PKCα and ζ translocation to the membrane fraction

FRTL-5 thyroid cells were incubated with TSH at different concentrations for 10 min as indicated. Membrane fractions were analyzed for PKCα and PKCζ by Western blotting. Representative Western blots for PKCα and PKCζ translocation to the membrane fraction are shown.

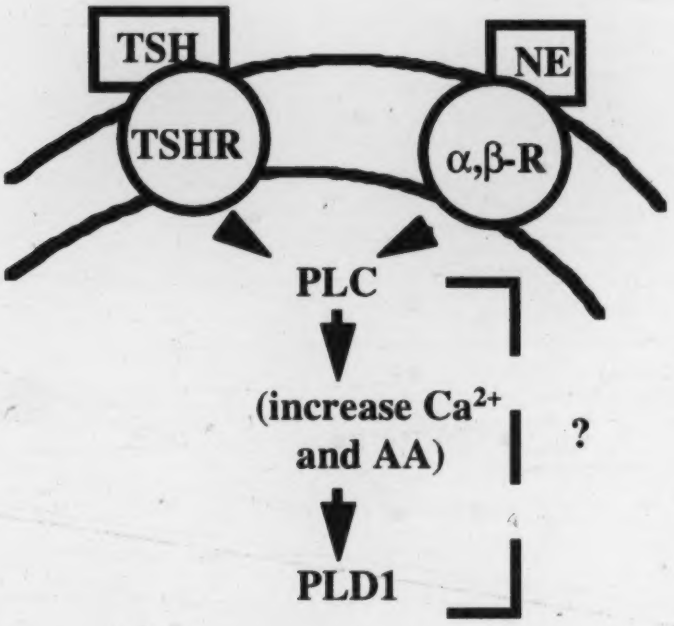

PKC α
0 10 30 100 1000
PKCζ
0 10 30 100 1000
TSH (μU/ml)

Thus, although PKC isoform translocation to the membrane was not observed in this study, longer incubation times may be required to observe the significant increases. The temporal activation of these PKC isoforms will be crucial to understanding how each isoform is involved in the pathways stimulated by TSH. In order to produce a more accurate picture of the events occurring intracellularly, molecular biology techniques, including the overexpression of the PKC isoforms will be performed and analyzed.

3.12 THE EFFECTS OF NOREPINEPHRINE ON PLD1 ACTIVATION IN FRTL-5 THYROID CELLS

Thyroid function can be regulated by a number of different signals. Regulators of thyroid hormone synthesis can act through stimulation of receptors to activate similar responses as TSH. The TSH receptor gene was cloned in 1989 (10-14) and has been studied since then in detail. The TSH receptor consists of two subunits, A and B, which share homology with other glycoprotein receptors (15). In fact, the A subunit is common to all glycoprotein hormone receptors (10-14). Although, some studies have shown that an eight amino acid tract near the amino terminus is an important site for receptor interaction with TSH (16), the exact nature of the binding site of TSH to its receptor has not been characterized.

Since the mechanism of TSH's ability to exert its effects is not completely understood, the effects of norepinephrine (NE) on iodide uptake were studied by Juvenal *et al.* (298) to determine if the sympathetic nervous system plays a role in increasing thyroid function. They found that NE alone acts through an α_1-adrenergic receptor to increase iodide uptake in FRTL-5 thyroid cells after 48 and 72 h (298). Although NE is an activator of both α- and β-adrenergic receptors, the conclusion that NE acts through only the α_1-adrenergic receptor in FRTL-5 thyroid cells after this long-term incubation is based on the observation that only an α_1-adrenergic receptor blocker was able to inhibit the effects of NE. Also, phenylephrine, a specific α_1-adrenergic activator, was able to mimic the effects of NE on iodide uptake (298). In addition to these observations, NE did not inhibit the ability of TSH to increase cAMP which implies that NE-mediated activities occur downstream of cAMP generation. Our lab wanted to investigate the ability of NE, acting through the α- or β-adrenergic receptor, to activate PLD1 in FRTL-5 thyroid cells to determine whether non-TSH-mediated PLD activation can occur.

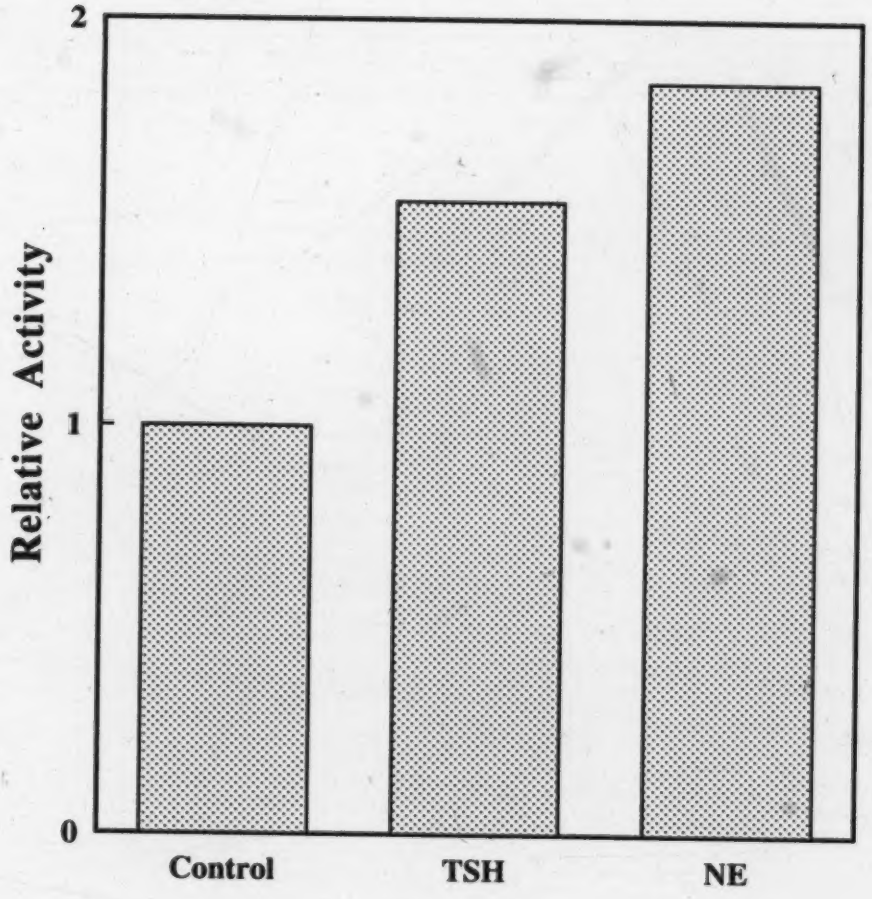


Preliminary results show that NE (5 μM) does activate PLD1 in the cell-free assay in a quantitatively similar fashion to TSH (Fig. 16). Activation of the α-adrenergic receptor stimulates an increase in intracellular Ca^{2+} and release of arachidonic acid (AA), which are both second messengers regulating a variety of thyroid functions (299,300). The β-adrenergic receptor is involved in increasing cAMP levels (301). The relevance of these observations in relation to FRTL-5 cells has not been fully investigated. Although the implications of this data are interesting and suggest that the α- and β-adrenergic receptors may play a role in regulating thyroid function, the exact nature of the regulation is unclear. The activation of PLD1 by NE could involve either of these receptors and their corresponding intracellular activities. This is the first study of short-term exposure of NE and its effect on PLD activity in FRTL-5 cells, thus, further studies will be required to distinguish between these two receptors and their influence

FIGURE 16

The effects of norepinephrine on PLD1 activation

FRTL-5 thyroid cells were incubated with TSH (100 μU/ml) or NE (5 μM) for 20 min prior to harvesting. The membrane fractions were used in the cell-free PLD assay in the presence of GTPγS. Results are expressed relative to the untreated control.


Relative Activity
2
1
0
Control
TSH
NE

on thyroid function.

3.13 THE EFFECTS OF HUMAN CHORIONIC GONADOTROPIN ON PLD ACTIVATION IN FRTL-5 THYROID CELLS

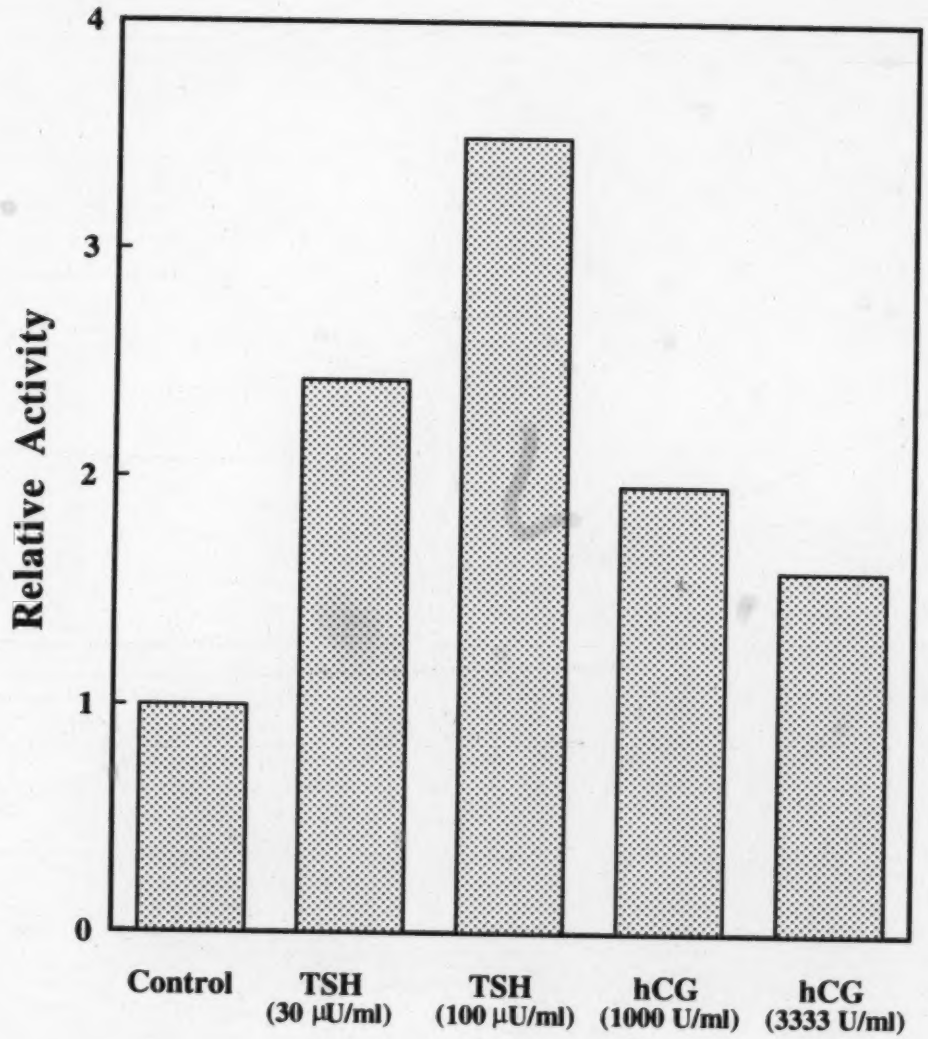
In addition to understanding the role of α_1-adrenergic receptors in PLD activation, the specific interaction between TSH and the TSH receptor is critical for connecting receptor function to its signal transduction cascade. In many cell types, several factors, including hormones, are able to stimulate PLD activity (56,69). In some patients with increased circulating levels of human chorionic gonadotropin (hCG) hyperthyroidism has also been observed (302). hCG is a glycoprotein hormone released by the placenta and is involved in the regulation and maintenance of pregnancy. Both TSH and hCG have α and β subunits with the α subunit being identical (11). Thus, the two hormones share approximately 70% homology (11). It has been demonstrated that hCG can interact with the TSH receptor and increase cAMP and iodide uptake (303). Thus, both hCG and TSH interact with the TSH receptor. The structure of the TSH receptor has been characterized, however, the mechanism of TSH binding is unclear. Similar regions between the hormone receptors could be shared critical active binding sites if hCG is shown capable of eliciting the same response in terms of PLD activation in FRTL-5 thyroid cells.

The transphosphatidylation reaction was used with FRTL-5 thyroid cells to determine the effect of hCG (1000 and 3333 μU/ml) on PLD activity. Preliminary data indicate that an increase in PLD activity was observed with hCG concentrations of 1000 and 3333 μU/ml (Fig. 17). These results are consistent with the concept that hCG activates PLD through the TSH receptor in FRTL-5 thyroid cells. It is not clear which pathway, either PKA or PKC, is mediating this effect. However, the use of specific PKA and PKC inhibitors should determine the involvement of each pathway in PLD stimulation by hCG. The exact nature of the interaction of TSH or hCG with the TSH receptor remains to be determined. However, the observation that hCG can activate

FIGURE 17

The effects of human chorionic gonadotropin on the activation of PLD

FRTL-5 thyroid cells were treated with [^{3}H]myristate for 2 h. Excess label was washed away and cells were treated with TSH (30 or 100 μU/ml), hCG (1000 or 3333 U/ml), or as controls for 30 min. Cells were scraped from tissue culture dishes with methanol and lipids were extracted. Lipids were run on TLC plates for separation and the PC and PEt bands were cut from plastic-backed silica gel plates. The activity from these bands was counted. PEt was calculated as a percentage of PC and these values were converted to relative activity as compared to the untreated control.


Relative Activity
4
3
2
1
0
Control
TSH
(30 μU/ml)
TSH
(100 μU/ml)
hCG
(1000 U/ml)
hCG
(3333 U/ml)

PLD in FRTL-5 thyroid cells lends support to the idea that hCG binds to the TSH receptor and activates similar intracellular pathways leading to PLD activation as TSH. The elucidation of shared critical amino acid regions and their modification may eventually provide a model for treatment of patients with hCG-induced hyperthyroidism.

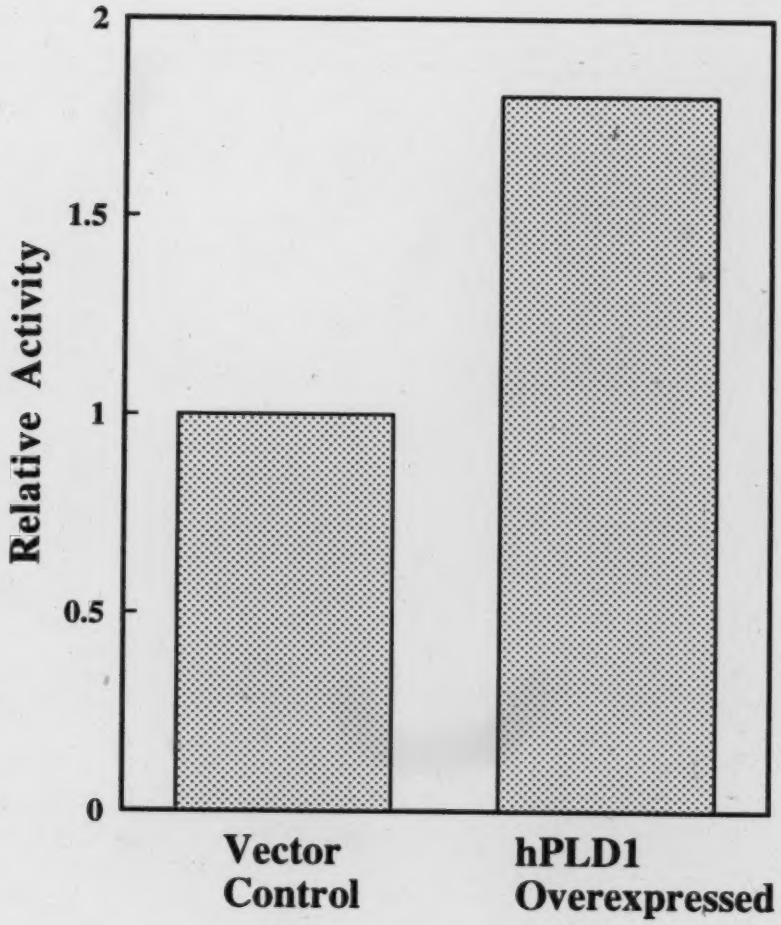
3.14 THE OVEREXPRESSION OF hPLD1 IN FRTL-5 THYROID CELLS

Our lab has shown that PLD1 is the predominant PLD isoform in FRTL-5 thyroid cells. PLD1 is activated by TSH and thus, is likely involved in the PKA and PKC pathways activated by TSH. In order to get a better understanding of the *in vivo* mechanisms of TSH action and to determine the effect that PLD1 has on thyroid function, molecular biological techniques were used to overexpress hPLD1 in FRTL-5 thyroid cells. FRTL-5 cells were transfected with human PLD1 cDNA for the overexpression of the hPLD1 enzyme by a postdoctoral fellow in the laboratory. The hPLD1 cDNA was kindly provided by Dr. Jeff Travers from the Indiana University School of Medicine, Indianapolis, USA. In order to determine if the transfection of hPLD1 was successful, a cell-free PLD assay was performed on the membrane fractions to determine if there was increased PLD1 activity. Cells containing the hPLD1 cDNA vector were assayed for PLD activity and compared to control cells infected with an empty vector. The results indicate that hPLD1 overexpressing cells treated with phorbol ester (PMA - 100 nM) in the presence of exogenous ARF (2 μM) showed almost a 2-fold increase over control cells containing the empty vector (Fig. 18). This indicates that these cells are overexpressing the hPLD1 enzyme. To establish the *in vivo* role of PLD1 in the regulation of thyroid function and growth, the phenotype of these hPLD1 overexpressed FRTL-5 cells will be characterized.

FIGURE 18

PLD activity in FRTL-5 thyroid cells overexpressing the human PLD1 enzyme

FRTL-5 thyroid cells were transfected with human PLD1 cDNA. Transfected and control cells were incubated with 100 nM PMA for 20 min and then harvested. The membrane fractions were used in the cell-free PLD assay in the presence of exogenous ARF (2 μM) and GTPγS. Results are expressed relative to the vector control transfected with the empty vector.


2
1.5
1
0.5
0
Relative Activity
Vector Control
hPLD1 Overexpressed

4. DISCUSSION AND FUTURE DIRECTIONS

The objectives of this project were to characterize the PLD activities in FRTL-5 thyroid cells and the role of TSH in stimulating PLD action. Since the intracellular regulation of thyroid hormone synthesis is not completely understood, this project attempted to elucidate the signalling pathways involved. The transphosphatidylation reaction and the cell-free assay were used to asses PLD activity in FRTL-5 thyroid cells in response to TSH and other agonists. The activation of identified small G-proteins known to be involved in PLD activation was also addressed in response to TSH stimulation. In addition, the relative contributions and regulation of different PLD isoforms by TSH was investigated. This is the first report of the regulation of two distinct pathways, PKA and PKC, by TSH which can activate PLD1 in FRTL-5 thyroid cells.

The initial studies involved the demonstration that LPA could activate PLD in FRTL-5 thyroid cells using the transphosphatidylation reaction. Previous studies have shown the activation of PLD by LPA (61) and the results in FRTL-5 thyroid cells were consistent with these findings. It is not clear from these studies which pathway(s) are involved in this PLD activation. However, further work from our lab may provide some insight. LPA has been shown to inhibit adenylyl cyclase in other cells (258). Thus, the generation of cAMP was tested in FRTL-5 thyroid cells in response to TSH in the presence of LPA by a postdoctoral fellow in the laboratory. The results from these experiments showed that LPA inhibited TSH-mediated cAMP generation, consistent with findings from other cell systems (290). However, from our proposed model of TSH action (section 1.9), an inhibition of cAMP generation would indicate a decrease in PLD activity. Our findings appear to contradict the proposed model. However, there is some data which may provide a means of reconciliation. The inhibition of cAMP generation by LPA occurred at both 10 and 50 μM LPA

concentrations (290). From the PLD studies, LPA was only able to activate PLD at 50 μM and not at 10 μM. Thus, the activation of PLD and the inhibition of cAMP generation by LPA are difficult to correlate. In other words, inhibition of cAMP does not appear to be PLD-mediated. Since different concentrations of LPA elicited different responses in terms of cAMP generation and PLD activation, the mechanism of action for these processes may be different. LPA acts by binding to its external *edg* receptor (258). *Edg* receptors are typically G-protein-coupled (258). These G-proteins can then directly inhibit adenylyl cyclase and inhibit cAMP generation (260,265). In addition to this action, LPA-induced G-protein activation can stimulate a number of additional intracellular pathways including the activation of PLC, tyrosine kinases and increasing intracellular Ca^{2+} (258,259). These effects can, in turn, lead to PLD activation. Thus, because LPA acts through its own *edg* receptor, its subsequent signalling pathways could mediate both the inhibition of adenylyl cyclase as well as downstream activation of PLD. Although the actions of LPA have not been fully characterized, our findings add some insight to the complex nature of the signalling pathways.

In addition to the cAMP and PLD responses to LPA, iodide uptake was also studied in FRTL-5 thyroid cells. Iodide uptake is a necessary step in thyroid hormone synthesis (6). These experiments were performed by a postdoctoral fellow in the laboratory and the effects of LPA were biphasic (290). Cells treated with 50 μM LPA for 24 h enhanced TSH-mediated iodide uptake by 23% ($P<0.03$, ANOVA). However, after 72 h, LPA (50 μM) inhibited TSH-mediated iodide uptake by 43% ($P<0.02$, ANOVA). The correlation of these results with those from the cAMP and PLD studies is difficult due to differences in the time courses used. However, LPA at 50 μM only leads to an initial increase in iodide uptake which corresponds to the increase in PLD activation by LPA also at 50 μM (290). Although there is not enough evidence to conclude that this PLD activation contributes to iodide uptake, the possibility is attractive as it demonstrates a logical sequence of events. Inhibition of

iodide uptake after long exposure (72 h) may represent desensitization of the receptor as observed with some other receptors. Further studies on the mechanism of action both upstream and downstream of PLD activity will be necessary to determine how these processes are regulated.

These results indicate the complex nature of the pathways within FRTL-5 thyroid cells. Activation of PLD by LPA does not appear to occur through the accumulation of cAMP. In fact, LPA itself acts to inhibit cAMP generation. Preliminary evidence revealed no effect of a PKC inhibitor on LPA inhibition of cAMP generation (290). Thus, the PKC pathway does not appear to be involved in LPA's inhibition of adenylyl cyclase. Since LPA can activate PLC giving rise to DAG and increased Ca^{2+} (258,259), PLD could be activated through activation of the PKC pathway, independent of the cAMP/PKA pathway. In addition to this possibility, LPA has been shown to induce tyrosine phosphorylation (272-274), which can also activate PLD. These are other possible mechanisms by which LPA could activate PLD. Although the mechanism of LPA action has not been completely elucidated, studies from other researchers will provide us with a path to further our own studies.

The above experiments have tested the effects of LPA added exogenously to cells and acting through an external *edg* receptor (258). Since the product of PLD action, PA, can be converted to LPA by PLA_2 (257), LPA may play an intracellular role which has not been investigated. These studies will require molecular biology for elucidation. The action of PLA_2 could be blocked or enhanced to determine the role LPA plays intracellularly. In addition to an intracellular role, LPA could also be released from thyroid cells to act in an autocrine or paracrine manner and thus, bind to its own external *edg* receptor or that of a neighbouring cell to elicit the responses described above. The role of LPA in thyroid function may involve secretion and binding to the *edg* receptor to stimulate a cascade of intracellular events. Further investigation into this possible role for LPA is needed. The effects of LPA on PLD

activity and iodide uptake will require further study of a number of intracellular steps and the results may help to clarify intracellular thyroid function and the possible communication between individual thyroid cells.

To further our understanding of the mechanism of TSH action on PLD in thyroid cells, the cell-free PLD assay was performed on the cytosolic, membrane and combined fractions. Since the maximal activation of PLD was dependent on GTPγS and the presence of cytosolic components, presumably ARF and RhoA, we determined that PLD1 is the predominant PLD isoform in FRTL-5 thyroid cells. In addition to this, the PLD assay performed in the presence of GDPβS, which inhibits G-proteins and, thus, PLD1, represents PLD2 and only about 20% of the total PLD activity present, with PLD1 comprising about 80%. This result is similar to the finding that PLD1 was the predominant isoform in HL60 cells (304). Since these results demonstrate the predominance of PLD1, this implicates not only the involvement of small G-proteins, but also the cellular events associated with the activation and translocation to the membrane fraction of those proteins. With this knowledge, investigations into the occurrence of events, such as actin cytoskeletal reorganization and secretory vesicle formation, may help to define the processes in thyroid cells which lead to thyroid hormone production and release. The G-protein ARF is involved in vesicular transport (76). The release of thyroid hormones from thyroid cells may involve the movement of thyroid hormone-containing vesicles to the cell surface for release into circulation. This process could be ARF-mediated. G-proteins of the Rho family are involved in cytoskeletal reorganization (77). This implicates Rho with a role in changes in cell shape, including the formation of pseudopods. When thyroid hormones are released, pseudopods extend from the thyroid follicular cells and release thyroid hormones upon fusion with a phagosome (1). Rho proteins could likely play a role in this process. The involvement of ARF and RhoA in TSH-mediated PLD1 activation has been demonstrated in this thesis. However, ARF and RhoA involvement

in the release of thyroid hormones has yet to be investigated. The role of these G-proteins in other cell systems (76,77) lends some support to the theory that ARF and RhoA may play similar roles in thyroid cells.

The identification of the ratio of PLD1 to PLD2 using varying PIP_2 concentrations demonstrates that both isoforms are present and, therefore, each PLD isoform has the potential to affect thyroid function in FRTL-5 thyroid cells. These experiments also demonstrate the requirement of both PLD isoforms for PIP_2. Having identified that both isoforms are present, it was necessary to determine which isoform(s) are activated by TSH. With the use of the Triton assay to inhibit PLD1 activity, we were able to measure PLD2 activity specifically. Many studies have been performed on the regulation of PLD2 and it has been shown in a number of them that PLD2 is constitutively active and unresponsive to external stimuli (79). Although Lopez *et al.* (81) suggested that PLD2 may be stimulated by ARF, these studies are not without limitations. While, the stimulation of PLD2 by exogenous ARF was about 2-fold and statistically significant compared to control, PLD1 activation increased by 20-fold. More importantly, the assay used to measure PLD2 activity was not specific to this enzyme and does not preclude activation of PLD1. Thus, it remains uncertain as to the role of ARF in the activation of PLD2. Another group studied a deletion mutant of PLD2 which showed that a deletion of the amino terminal end of the protein resulted in an increased response to ARF (84). This phenomena has yet to be explained, however some have implicated this region in inhibiting ARF interaction with PLD2. In fact, there is some suggestion that, *in vivo*, PLD2 may be cleaved to allow it to respond to ARF (84). Further studies with PLD2 dominant negative splice variants also implicate ARF in insulin-mediated PLD2 activation (156). However, the results from this study depend on the down-regulation of PLD enzymes in response to their respective splice variants. Although in some cases this technique may prove useful in identifying the role of some proteins, in this study there is no evidence that these splice variants do not

interact with both isoforms nor has the mechanism of their action been elucidated. Thus, it is premature to speculate , from these studies, that the physiological activation of native PLD2 involves ARF.

In spite of some evidence of a role for ARF in PLD2 activation *in vivo*, there has been no consistent evidence that PLD2 is regulated by ARF *in vitro*. In addition, significant evidence exists to the contrary in that PLD2 is unresponsive to ARF (79). Therefore, in FRTL-5 thyroid cells, with the use of the cell-free PLD assay *in vitro*, we demonstrated PLD2 independence of ARF. Using the PLD assay with varying concentrations of PIP_2 (section 3.4), the observation of some PLD activity in the presence of GDPβS demonstrates a G-protein-independent PLD activity, namely PLD2, in FRTL-5 thyroid cells. Thus, based on this and evidence from several other studies (79), PLD2, *in vitro*, appears independent of the small G-proteins ARF and Rho. In light of this evidence and to confirm that TSH was not activating PLD2, the Triton assay was used to measure PLD2 activity specifically in response to TSH. Concentrations of TSH up to 1 mU/ml were unable to activate PLD2 which is consistent with other groups who found PLD2 was unresponsive to agonist stimulation (79). Thus, we concluded that PLD2 is unlikely to be involved in TSH-mediated PLD activation in FRTL-5 thyroid cells, although we cannot completely exclude the possibility.

Since PLD2 does not appear to be activated by TSH in FRTL-5 thyroid cells, the effect of TSH on PLD1 was investigated. After a 20 min incubation with TSH at 100 μU/ml, PLD was maximally activated by greater than 2-fold above control values in the membrane fraction. This PLD1 activation was dependent on GTPγS. This result is consistent with findings from other groups who have shown that PLD1 is responsive to external agonists (56,69). In HIRcB fibroblast cells overexpressing the human insulin receptor, treatment with insulin causes an increase in PLD activity that is primarily ARF-mediated (157). In adipocytes, insulin activates a Rho-mediated PLD

activity (158). In addition to these, agonists known to activate PKCα or bind G-protein-coupled receptors, like PMA and fMLP respectively, also activate PLD activity (56,69). Since we have shown that TSH does not activate PLD2 in FRTL-5 thyroid cells, it is reasonable to assume that the PLD activity observed after stimulation with TSH is exclusively PLD1. These results provide evidence of a possible role for PLD1 in the two pathways activated by TSH, namely the PKA and PKC pathways, as well as small G-proteins, like ARF and RhoA, in TSH action.

The dependence of PLD1 on small G-proteins has been studied in many cells (56,69). Thus, we focused on the role of small G-proteins in the activation of PLD1 in response to stimulation by TSH. To determine the involvement of ARF and RhoA in TSH-mediated PLD1 activation, the membrane content of these two proteins was measured with and without TSH stimulation. After a 10 min incubation with 100 μU/ml TSH, both ARF and RhoA were translocated to the membrane fraction, which indicates that both proteins could be involved in mediating the actions of TSH on PLD1 activation in FRTL-5 thyroid cells. Whether specific actions of ARF and RhoA play a role in non-PLD activities remains unclear. However, since the goal of TSH stimulation is the production of thyroid hormone, the small G-proteins may be involved in this action beyond merely activating PLD1 in a synergistic manner (91,92). The specific actions of each of these small G-proteins should be considered. For instance, as stated above, small G-proteins like ARF and Rho mediate other intracellular events including vesicle formation (76) and cytoskeletal reorganization (77), respectively. Although ARF and RhoA play a role in the activation of PLD1, they may also be involved in the activation of other processes regulating the secretion of stored thyroid hormones independent of PLD1 activation.

ARF proteins are involved in vesicular formation and trafficking in most eukaryotic cells (76). They play roles in the secretion of proteins and other cellular products (76), which could include the secretion of thyroid hormones from thyroid

cells. The action of ARF in vesicular trafficking may depend on its subcellular location and translocation to the membrane fraction where it is able to perform its function. Although the connection between ARF and thyroid hormone secretion has not been studied, the idea proposes a PLD-independent activity for ARF and provides another link between TSH action and the need for small G-protein activation.

Rho proteins typically act as molecular switches as they cycle between the active and inactive form (77). These proteins are involved in the reorganization of the actin cytoskeleton and in the initiation of gene transcription (77). The effects of Rho proteins often involve changes in cell shape and movement both within the cell and of the cell itself. The connection between these processes and thyroid hormone production has, again, not been studied. However, such cytoskeletal reorganization may be necessary for both the production and secretion of thyroid hormones. In addition, the activation of Rho may be involved in the transcription of genes which are necessary for the regulation of thyroid hormone production. Rho proteins have also been implicated with a role in the activation of NADPH oxidase in phagocytes (130) which leads to the production of H_2O_2, a necessary component of iodide organification for thyroid hormone synthesis (6). This could be a non-PLD related function for Rho proteins in thyroid cells. Although the role of ARF and RhoA have not been clearly identified in thyroid cells, it is clear that they play a role in the activation of PLD1 and, possibly, in events independent of PLD activation related to thyroid hormone production and secretion.

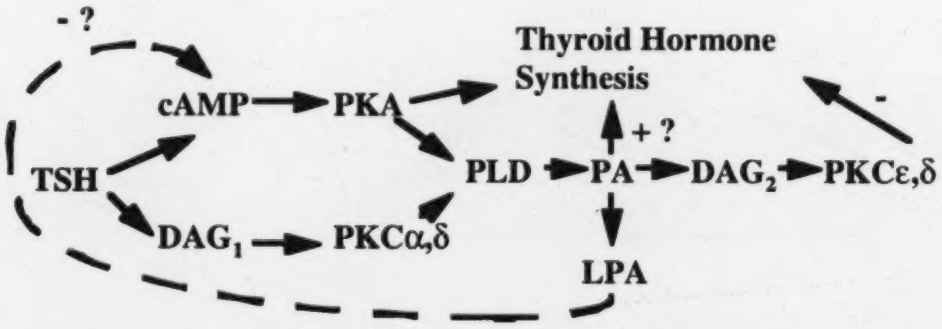
In order to establish the intracellular pathways by which TSH acts to stimulate PLD1, specific inhibitors to the PKA and PKC pathways were used. Either of the PKA and PKC inhibitors, DDA and chelerythrine respectively, were able to block TSH-mediated PLD1 activity. This suggests activation of both pathways is required for optimal TSH activation of PLD1 in FRTL-5 thyroid cells. However, the relative contributions of each pathway to the activation of PLD1 were difficult to establish with

this approach. Preliminary results showed that both PMA, an activator of PKC, and forskolin, an activator of PKA, were able to activate PLD in FRTL-5 thyroid cells to similar levels of PLD activation by TSH. Although the contribution of each pathway to this activation remains unclear, these results are consistent with the involvement of both pathways in TSH-mediated PLD activity.

Along with PLD activation, the relative contributions of the two pathways to small G-protein translocation was investigated. Preliminary results with RhoA showed increases in translocation to the membrane fraction after stimulation with PMA or dbcAMP, which is an activator of PKA. This suggests that RhoA translocation occurs via either pathway, but these results must be confirmed. Since the levels of translocation were similar to those observed with TSH, small G-proteins, like RhoA, may be involved in the activation of both pathways in thyroid cells. In addition, since Rho proteins may carry out additional roles in the cell besides the activation of PLD1, the stimulation of RhoA may involve its movement to other subcellular locations to carry out other duties. This has yet to be determined.

The role of the PKA pathway in thyroid hormone production has been known and studied for many years (15). Although the events leading to thyroid hormone production have been investigated, the role of the PKC pathway in thyroid hormone production is not entirely understood. PKC activators have been shown to inhibit the production of thyroid hormones (48). However, the mechanism by which this occurs is unknown. Both the PKA and PKC pathways have been shown to converge on the activation of PLD1 in FRTL-5 thyroid cells (53,55). Only the PKCα and β isoforms have been shown to activate PLD (238-240), with some recent evidence that the PKCδ isoform may also be able to do so (241). The role of different PKC isoforms has not been clearly identified in thyroid cells. Since different isoforms have been shown to elicit isoform-specific responses in other cells (72), it is likely that a similar result will be found in thyroid cells. PKC is activated by DAG (72). The generation of DAG by

different enzymes leads to the production of different DAG species (64). For instance, DAG derived from PLC activity is different from that derived from PLD activity via PA (191). Due to the differences in DAG fatty acid composition which is derived from different sources, PKC isoforms can be activated at different times and by different factors in the cell (64). Although inhibition of thyroid hormone synthesis by PKC occurs, the exact PKC isoform(s) involved are not known. In addition, the time and place of the inhibition is also unknown. Thus, the temporal activation of different PKC isoforms will be the essential key to determining the complete role of PKC in the FRTL-5 cell. In section 1.14 , an outline of the possible role of PKC isoforms was presented. Further definition of this model is shown below with the inclusion of the possible presence and action of different PKC isoforms.



PKCζ is also present in FRTL-5 thyroid cells and it has been shown to activate PA in some systems (184). Although that has not been established in FRTL-5 cells, it is a possibility. The specific role of each PKC isoform will require transfection of PKC isoforms into FRTL-5 cells to determine their temporal response to TSH stimulation.

In addition to the study of the intracellular pathways involved in thyroid function, the TSH receptor has been studied in some detail. The structure of the TSH receptor has been postulated, although the site of interaction with TSH has not been clearly defined (15). The effects of NE on PLD activation were studied in FRTL-5 thyroid cells to determine if adrenergic receptors plays a role in actions attributed to

TSH. Preliminary results demonstrated that NE activated PLD to a level similar to that of TSH. NE can act through α- or β-adrenergic receptors (299-301). *In vivo* studies have shown that α-receptors inhibit thyroid function while β-receptors stimulate TSH-induced T_4 release (299-301). Juvenal *et al.* (298) showed that NE after long exposures (48 and 72 h), but not after 24 h, inhibited the ability of TSH to induce iodide uptake. NE inhibited the ability of dbcAMP to increase iodide uptake but did not alter cAMP levels increased by TSH. Thus, NE appears to induce its effect downstream of cAMP generation. In addition, only the α_1-adrenergic blocker prazosin, and not a β-adrenergic blocker, was able to inhibit NE action in FRTL-5 cells. Phenylephrine, a specific α_1-adrenergic agonist, mimicked the effects of NE. These data are consistent with NE acting through the α_1-adrenergic receptor after long exposure times, although more evidence is needed to confirm this.

α_1-Adrenergic receptors act by increasing inositol-trisphosphate (IP_3) and Ca^{2+} concentrations (299,300), which can activate the PKC pathway. β-adrenergic receptors act by increasing cAMP levels (301). TSH has been shown to upregulate α_1-adrenergic receptors in the thyroid gland *in vivo* (305), and in FRTL-5 thyroid cells (306). However, evidence from other groups showed that 70% of the maximal response to TSH occurs at 24 h and the complete response at 48 h (307). Since NE did not exert its effect until 48 h in the Juvenal study, this implies the participation of another unidentified molecule in this process. Although one group reported reduced TSH-mediated cAMP generation after epinephrine treatment (308), Grollman *et al.* showed no effect of NE on this parameter (309). The NE study in this thesis attempted to determine the acute effects of NE on adrenergic receptor stimulation and PLD activation. Further studies with more specific receptor agonists, such as phenylephrine, a specific activator of α_1-adrenergic receptors, as well as activators of β-receptors will help to identify the acute mechanism of adrenergic receptor activation by NE in thyroid cells.

To further understand the role of receptor action in mediating effects in FRTL-5 thyroid cells, the effect of hCG on PLD activation was studied. Since the observation that patients with increased circulating levels of hCG associated with choriocarcinoma exhibited hyperthyroidism (302), it has been thought that hCG itself was thyrotropic as hCG has been shown to interact with the TSH receptor (303). To confirm this hypothesis, PLD activity was tested in response to hCG stimulation. Preliminary results indicate that hCG is able to activate PLD in FRTL-5 thyroid cells. These results provide evidence that hCG is acting via the TSH receptor and is capable of PLD activation. The pathway by which this occurs has not been confirmed, however, some studies have shown that hCG is capable of increasing cAMP levels (303). Crystallization studies for both TSH and hCG interaction with the TSH receptor or TSH receptor deletion mutants may be necessary to characterize the differential interaction of both hormones with the TSH receptor.

The overexpression of the hPLD1 cDNA into FRTL-5 cells in our lab has carried this project into the molecular biology era where the *in vivo* effects of TSH action can be studied. Human PLD1 was overexpressed in FRTL-5 thyroid cells by a postdoctoral fellow in the lab and PLD1 activity was shown to be increased in response to PMA in the presence of exogenous ARF when compared to cells transfected with an empty vector alone. These molecular tools should allow for the elucidation of the role of PLD1 in the regulation of differentiated thyroid function. Further molecular techniques will be used to establish the role of other factors involved in these pathways.

We have added to the further understanding of TSH action in thyroid cells. The involvement of two separate pathways and the presence of different isoforms of key components in the pathways only serves to increase the complexity of the system. The current studies have demonstrated the predominance of the PLD1 isoform in FRTL-5 thyroid cells which is activated by TSH through the translocation of the small G-proteins, ARF and RhoA, to the membrane fraction. Both the PKA and PKC

pathways are activated by TSH and appear to converge on PLD1 activation. Beyond the activation of PLD1, the activities of the products of PLD action are not fully understood. However, we have presented a model which incorporates many of the findings of other labs in relation to the function of signalling molecules such as PA, LPA and DAG. This model may represent some of the intracellular regulation that occurs in FRTL-5 thyroid cells. With the addition of molecular biological tools to determine the role of specific proteins in the pathway, we can identify the specific function of proteins like PKC and other factors which will give us a clearer picture of the inhibitory signal in thyroid cells. Elucidating these aspects of thyroid function will provide the means to effectively understand and treat thyroid disease.

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